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ORIGINAL RESEARCH

RETRACTED ARTICLE: Dihydrotanshinone I inhibits the growth of osteosarcoma through the Wnt/β-catenin signaling pathway

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Background: Osteosarcoma is a common malignant tumor, with relatively lower survival rates in adolescents. Dihydrotanshinone I (DHI) was extracted from the traditional Chinese medicine *Salvia miltiorrhiza* and was shown to inhibit several types of cancer.

Purpose: To explore the effect of DHI on the proliferation, migration, invasion, and apoptosis of osteosarcoma cells, as well as its possible molecular mechanism.

Methods: The effect of DHI on the proliferation of osteosarcoma was detected by crystal violet assay, MTT assay, colony formation assay. The effects of DHI on the migration and invasion of osteosarcoma were detected by wound healing assays, cell migration and invasion assays. The effect of DHI on apoptosis of osteosarcoma was detected by cell apoptosis assay and Hoechst apoptosis staining. The protein expression levels were detected by Western blotting assay. The activity of Wnt/β-Catenin signaling pathway was detected by luciferase reporter assay and western blot. The inhibitory effect of DHI on osteosarcoma in vivo was analyzed using an orthotopic OS tumor animal model and immunohistochemistry.

Results: DHI may inhibit the proliferation, decrease the migration, reduce the invasion, and promote the apoptosis of osteosarcoma cells. In vivo mouse model, DHI can inhibit the formation of osteosarcoma. In terms of mechanism, DHI may inhibit both the transcriptional activity and the total protein level of β-catenin.

Conclusion: DHI may inhibit the proliferation, migration, and invasion as well as induce the apoptosis of osteosarcoma cells, possibly through suppressing the Wnt/β-catenin signaling pathway.

Keywords: dihydrotanshinone I, osteosarcoma, Wnt/β-catenin signalling pathway, proliferation, migration, invasion

Introduction

Osteosarcoma (OS) is one of the most common malignant tumors of the bone; OS develops from the interstitial cell line. The rapid growth of the tumor, which can easily metastasize to the lung and pleura, is due to the formation of osteoid tissue and bone tissue directly or indirectly through the cartilage stage. The main clinical treatments for osteosarcoma patients are extensive resection of primary tumors and chemotherapy.[1,2] At present, drugs such as doxorubicin, cisplatin, high-dose methotrexate, and isocyclophosphamide are used in the treatment of osteosarcoma, which not only make many patients with osteosarcoma intolerant of drug toxicity[1] but also cause hemolysis,[3] damage of liver and kidney function,[4] and even endanger patients' lives. Therefore, it is necessary to explore safer and more

effective drugs for malignant osteosarcoma cells. Several studies have evidenced that Chinese medicine has a good preventive or therapeutic effect on various malignant tumors. Dihydrotanshinone I (DHI) is a traditional Chinese medicine extracted from Salvia miltiorrhiza of Radix Salviae Miltiorrhizae. Since Lee D.S et al[5] reported in 1999 that DHI inhibited K562 leukemic cells. A large number of studies have confirmed that DHI has profound effects against human tumors, including breast cancer,[6] colon cancer,[7] gastric cancer,[8] glioma,[9] and hemangioma. Yihong Cai et al[10] compared the 14 main compounds extracted from Tanshen (the Chinese name of Salvia miltiorrhiza) and found that DHI is the most effective regulator for inhibiting hemangioma. In terms of potential mechanisms, DHI has effects on JNK and Fasl signaling pathways, endoplasmic reticulum stress, and activation of ATF3, through which tumor cells are inhibited. Despite these meaningful findings, the exact molecular mechanisms by which DHI exerts its anticancer effects remain to be fully investigated.

In this present study, we investigated the antitumor effect of DHI on human OS cells, as well as the possible molecular mechanism involved. Our results intensively indicated that DHI may inhibit the growth of osteosarcoma cells in vitro and in vivo. Moreover, the inhibitory effect of DHI on OS cells may be mediated by suppression of Wnt/β-Catenin.

Materials and methods

Cell culture and drug preparations

OS cell lines 143B, U2OS, Saos2, and MG63 were obtained from the American Type Culture Collection (ATCC, USA), and maintained in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, USA) with 10% fetal bovine serum (FBS, Excell biology, China), 100 μg/mL of streptomycin and 100 U/mL of penicillin at 37°C in 5% CO_2. DHI was purchased from Chengdu Must Bio-purify Co. Ltd (China) and was dissolved in dimethyl sulfoxide (DMSO).

Crystal violet assay

OS cells were seeded in 24-well plates and treated with different concentrations of DHI (2, 4, or 6 μM) or DMSO, as a control, for 24, 48, or 72 hrs. Cells were stained with crystal violet to visualize the cell viability. For quantification, 24-well plates were stained with crystal violet, and 200 μL of 10% acetic acid was added to each well. The plates with cells were placed in multifunctional enzyme labeling instrument to detect the absorbance at 595 nm.[11] Cell viability was calculated using the following formula: cell viability (%)=experimental group absorbance value/0 μM group absorbance value×100%. All assays were performed in triplicate.

MTT assay

OS cells were seeded into 96-well plates at a density of 5,000 cells/well and incubated for 12 hrs for adherence. To determine the individual effects of DHI on OS cells, cells were incubated with various concentrations of DHI (2, 4, or 6 μM) or DMSO, as a control, for 24 or 48 hrs. Following the different treatments, 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, USA) was added to each well, and the plates were incubated at 37°C for 4 hrs. Then, MTT was removed and 150 μL/well DMSO was added. Plates were vibrated for 20 mins on the shaking table. The plates with cells were subsequently placed in a multifunctional enzyme labeling instrument to detect the absorbance at 490 nm. All assays were performed in triplicate.

Colony formation assay

Osteosarcoma cells were plated into 6-well plates (1×10^2 cells/plate) and incubated in DMEM or DHI supplemented with 10% FBS at 37°C. After 1 week, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 mins, stained with crystal violet, and the number of colonies was counted. All assays were performed in triplicate.

Wound healing assays

We seeded 143B cells into 6-well plates. The confluent monolayer was scratched by the tip of a 10 μL pipette in order to create a cell-free region, and then the cells were cultured in 2% FBS medium with DHI (2, 4, or 6 μM) or DMSO, as a control. At the indicated time points, three different fields of each wound were randomly photographed using a light microscope.

Cell migration and invasion assays

Cell migration and invasion were detected by Transwell experiments. For cell invasion assay, the upper chamber of the Transwell (Corning, NY, USA) was coated with Matrigel (BD Biosciences) at 37°C in a 5% CO_2 incubator for 30 mins. OS cells were trypsinized, cultured in serum-free DMEM medium with DHI (2, 4, or 6 μM) or DMSO, as a control, and placed in the upper chamber (4×10^4 cells/well). Then, 10% FBS was added to the lower chamber.

The plates were incubated for 24 hrs. Cells in the upper chamber were completely removed with a cotton swab. Cells migrating into the lower chamber were washed with PBS, fixed in 4% paraformaldehyde, and stained with crystal violet. Finally, the cells were counted under a microscope in three random fields. For cell migration assays, the experiments were performed under the same conditions, except Matrigel was removed when the upper chamber was precoated.[12]

Cell apoptosis assay

Cells were seeded into 60 cm culture dishes and treated with different concentrations of DHI (2, 4, or 6 μM) or DMSO, as a control, for 24 hrs. Then, the cells were collected, washed twice with ice-cold phosphate-buffered saline (PBS), and stained with Annexin V-FITC and PI according to the manufacturer's guidelines. The samples were then read on a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The distribution of viable (FITC-/PI-), early apoptotic (FITC+/PI-), late apoptotic (FITC+/PI+), and necrotic (FITC-/PI+) cells was analyzed. Both early and late apoptotic cells were recorded as apoptotic cells, and the results are expressed as the percentage of total cells.[13]

Hoechst apoptosis staining

We plated 143B cells in 24-well plates and treated them with different concentrations of DHA (2, 4, or 6 μM) or DMSO, as a control, for 24 hrs. Cells were collected and stained with Hoechst 33258. Pictures were taken under a fluorescence microscope.

Mitochondrial membrane potential assay

Cells were seeded into 24-well plates and treated with different concentrations of DHI (2, 4, or 6 μM) or DMSO, as a control, for 24 hrs. Cells were washed with PBS and incubated in medium containing 250 μL Preparation of JC-1 working fluid (Beyotime Institute of Biotechnology, China) at 37°C for 20 mins. After washing with ice-cold JC-1 buffer, medium was added. The cells were counted under a fluorescence microscope in three random fields.[13]

Luciferase reporter assay

We seeded 143B cells in T-25 flasks and transfected them with 4 μg of Top-luc luciferase reporter plasmids. Twenty-four hours later, transfected osteosarcoma cells were seeded to 24-well plates and treated with DHI (2, 4, or 6 μM) or DMSO, as a control, for 12 hrs. At the scheduled time points, cell supernatants were treated by luciferase assay kit, and the level of luciferase expression was detected by GloMax luminescence detector (Promega Company, USA). Each assay was performed in triplicate.[14]

Establishment of orthotopic OS tumor animal model

The 143B suspension (2×10^7 cells/ml) was injected into the proximal tibia of 4–6 weeks old female mice. Then, animals were treated with different doses of DHI (5, 15, or 25 mg/kg) or sodium carboxymethyl cellulose (CMC-Na), as a control, once every 2 days. The tumor length and width were measured every 2 days after the first week, and the animals were killed 21 days after injection. The tumor volume was calculated according to the following formula: $0.5\times L\times W^2$ (L is tumor length; W is tumor width). The specimens of tumor tissues were collected for follow-up tests. All animal experiments were approved by (IACUC) of animal protection and utilization organization committee of Chongqing Medical University. This housing facility is a barrier housing facility, and it has in keeping with national standard "Laboratory Animal—Requirements of Environment and Housing Facilities" (GB 14,925—2010). The care of laboratory animal and the animal experimental operation have conforming to "Chongqing Management Approach of Laboratory Animal" (Chongqing government order NO.195).

Western blot assay

The 143B cells treated with different concentrations of DHI and DMSO, as a control, for 24 hrs. Then, cells were collected and dissolved in the protein cleavage solution for protein extraction. The protein concentration was detected by BCA kit, and proteins were separated by 6–15% gradient SDS-PAGE. After separation, the protein was transferred onto PVDF membranes, which were then blocked with 5% FBS in TBST for 2 hrs. Primary antibody dilution buffer was used to incubate PVDF membranes overnight at 4°C. Anti-rabbit IgG, HRP-linked antibody was used to incubate PVDF membrane at 37°C for 1 hr. The specific protein bands were visualized using an ECL kit (Millipore, USA).[15]

Immunohistochemistry (IHC)

Immunofluorescence was employed to show PCNA, Bcl-2, N-Cadherin, and β-Catenin expression in tumor tissue. The tumor tissues section was blocked and immunostained

with antibodies targeting PCNA (1:100), Bcl-2 (1:100), N-Cadherin (1:50), and β-Catenin (1:100). The picture was captured using a light microscope. The mean of integrated optical density (IOD) was detected by Image-Pro Plus 6.0 software in three random fields.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences between 2 groups were assessed using two-tailed Student's *t*-tests. Each experiment was performed at least 3 times. The results are displayed as the mean±SD. A *P*-value <0.05 was considered statistically significant.

Results

DHI inhibits the proliferation of OS cells

First, the effects of DHI on the proliferation of OS cells were detected by crystal violet staining. We found that DHI inhibited the proliferation of 143B and MG63 OS cells in a concentration-dependent manner (Figure 1A–D, $P<0.05$). Similar results were obtained in two other commonly used OS cell lines, SaoS2 and U2O (Figure 1E and F, $P<0.05$). The inhibitory effect of DHI on the proliferation of 143B cells and MG63 cells was further verified by MTT assay (Figure 1G and H, $P<0.05$) and colony formation assay (Figure 1I–L, $P<0.05$). Moreover, we found that DHI suppressed the protein level of PCNA (Figure 1M and N, $P<0.05$), which is a well-established marker for cell proliferation. Collectively, these results suggest that DHI may effectively inhibit the proliferation of OS cells.

DHI inhibited the migration and invasion of OS cells

We next assessed the effect of DHI on the migration ability of OS cells. Using wound healing assay, we found that DHI inhibited the ability of 143B and MG63 cells to close the wound gap. (Figure 2A–D, $P<0.05$). Next, using Transwell assay without ECM, we found that DHI decreased the number of migrated OS cells in a dose-dependent manner (Figure 2E–H, $P<0.05$). Then, we determined the invasive potential of OS cells using Matrigel Transwell assay. We found that DHI significantly inhibited the migration of 143B and MG63 cells, leading to a decrease in the number of perforated cells (Figure 2I–L, $P<0.05$). Moreover, we determined by Western blot that DHI reduced the expression of MMP9, MMP2, MMP-7, Snail, and N-Cadherin (Figure 2M and N, $P<0.05$), which are potent markers for tumor metastasis and invasion. Together, these results suggest that DHI may inhibit the migration and invasion of OS cells.

DHI promotes the apoptosis of osteosarcoma cells

To evaluate the effect of DHI on apoptosis of 143B and MG63 cells, we detected the apoptosis of 143B and MG63 cells by flow cytometry (Figure 3A–D, $P<0.05$). The results showed that early and late apoptosis of 143B and MG63 cells increased significantly. The results of Hoechst 33258 staining further showed that DHI could promote the apoptosis of 143B and MG63 cells in a concentration-dependent manner (Figure 3E–H, $P<0.05$). These results suggest that DHI can promote the apoptosis of osteosarcoma cells. DHI increased the expression of PARP, Cleaved PARP, Cleaved Caspase-3, and Caspase-3. At the same time, DHI also reduced the expression of Bcl-2 (Figure 3I and J, $P<0.05$). To further explore whether DHI acts through the mitochondrial apoptosis pathway, we conducted a JC-1 test. The results showed that the amount of red fluorescence decreased and the green fluorescence increased with the increase of DHI concentration (Figure 3K, $P<0.05$). These results suggest that DHI may induce the apoptosis of osteosarcoma cells through the mitochondrial apoptosis pathway.

DHI inhibits the Wnt/β-catenin signaling pathway in osteosarcoma cells

We have demonstrated that DHI can inhibit osteosarcoma cells in vitro. Next, we explored the possible mechanism of the anticancer effect of DHI in OS cells. We found that DHI reduced Top-luc and c-myc-luc by using a luciferase reporter gene system (Figure 4A and B, $P<0.05$). TOP-Luc contains TCF/LEF-responsive elements and reflects β-catenin transcriptional activity; c-Myc-Luc contains c-Myc-responsive elements and reflects c-Myc transcriptional activity. These results indicate that DHI suppresses Wnt/β-catenin signaling and its downstream molecule c-Myc in OS cells. To investigate whether DHI inhibits the Wnt/β-Catenin signaling pathway at the protein level in OS cells, we carried out Western blot assays. The results showed that DHI treatment downregulated the expression of β-Catenin, LRP6 (upstream of β-Catenin), c-Myc (downstream of β-Catenin), and Cyclin D1 proteins (Figure 4 Cand D, $P<0.05$). These results suggest that DHI can inhibit the Wnt/β-Catenin signaling pathway in osteosarcoma cells.

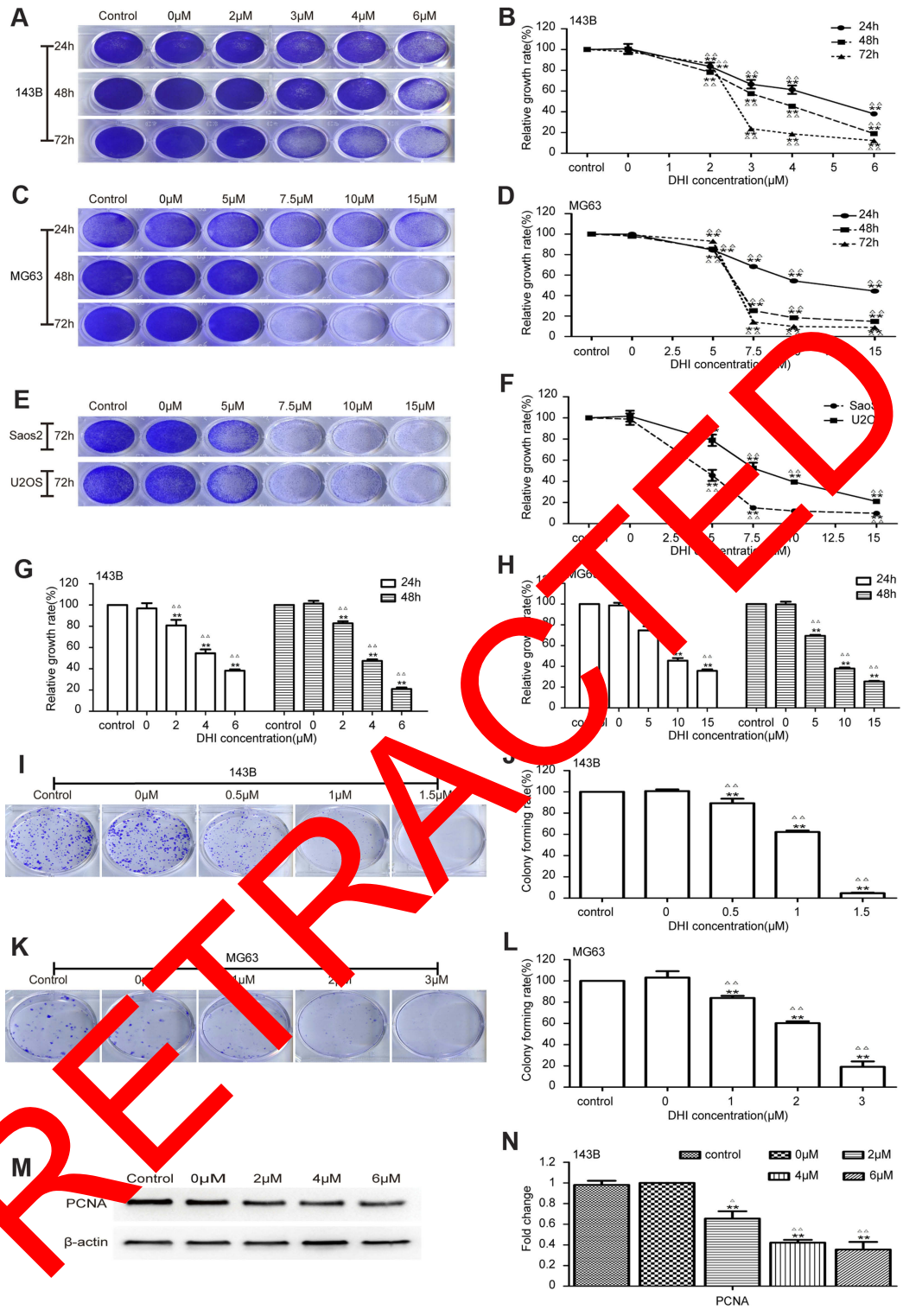

Figure 1 DHI inhibited OS cell proliferation in vitro.

Notes: (**A**–**F**) DHI suppressed OS cell proliferation, as measured by crystal violet staining. (**G** and **H**) DHI suppressed OS cell proliferation, as measured by the MTT assay. (**I**–**L**) Colony formation assays showed a significantly lower colony forming rate in both cell lines following DHI treatment. (**M** and **N**) Western blot analysis showing DHI downregulated PCNA. μM: The different concentrations of DHI; Control: Osteosarcoma 143B cells were treated with DMSO to exclude the effect of DMSO on the test. $^{\Delta}P<0.05$, vs the control group; $^{\Delta\Delta}P<0.01$, vs the control group; $^{**}P<0.01$, vs 0 μmol/L DHI group (n=3).

Abbreviations: DHI, Dihydrotanshinone I; OS, osteosarcoma.

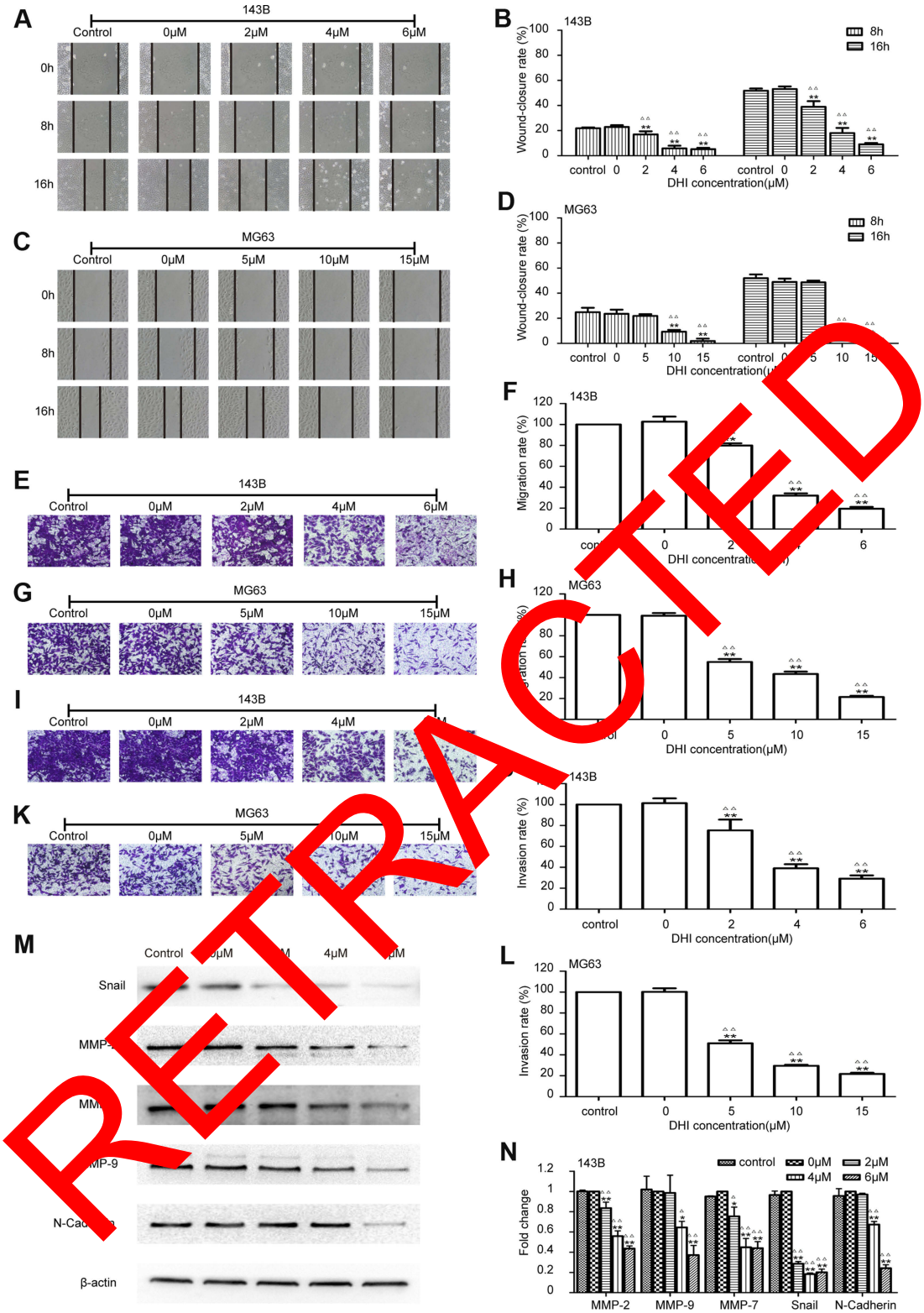

Figure 2 DHI inhibited OS cells migration and invasion in vitro.

Notes: (**A**–**D**) Wound healing assays showed significantly decreased migratory abilities in both cell lines following DHI treatment. (**E**–**H**) Transwell assays showed significantly decreased migration abilities in both cell lines following DHI treatment. (**I**–**L**) Transwell assays showed significantly decreased invasive abilities in both cell lines following DHI treatment. (**M**-**N**) Western blot analysis showing DHI downregulated Snail, MMP-2, MMP-7, MMP-9, and N-Cadherin. μM: The different concentrations of DHI; Control: Osteosarcoma 143B cells were treated with DMSO to exclude the effect of DMSO on the test. $^{\Delta\Delta}P<0.01$, vs the control group; $^{**}P<0.01$, vs 0 μmol/L DHI group (n=3).

Abbreviations: MMP, Matrix metalloproteinase; DHI, Dihydrotanshinone I.

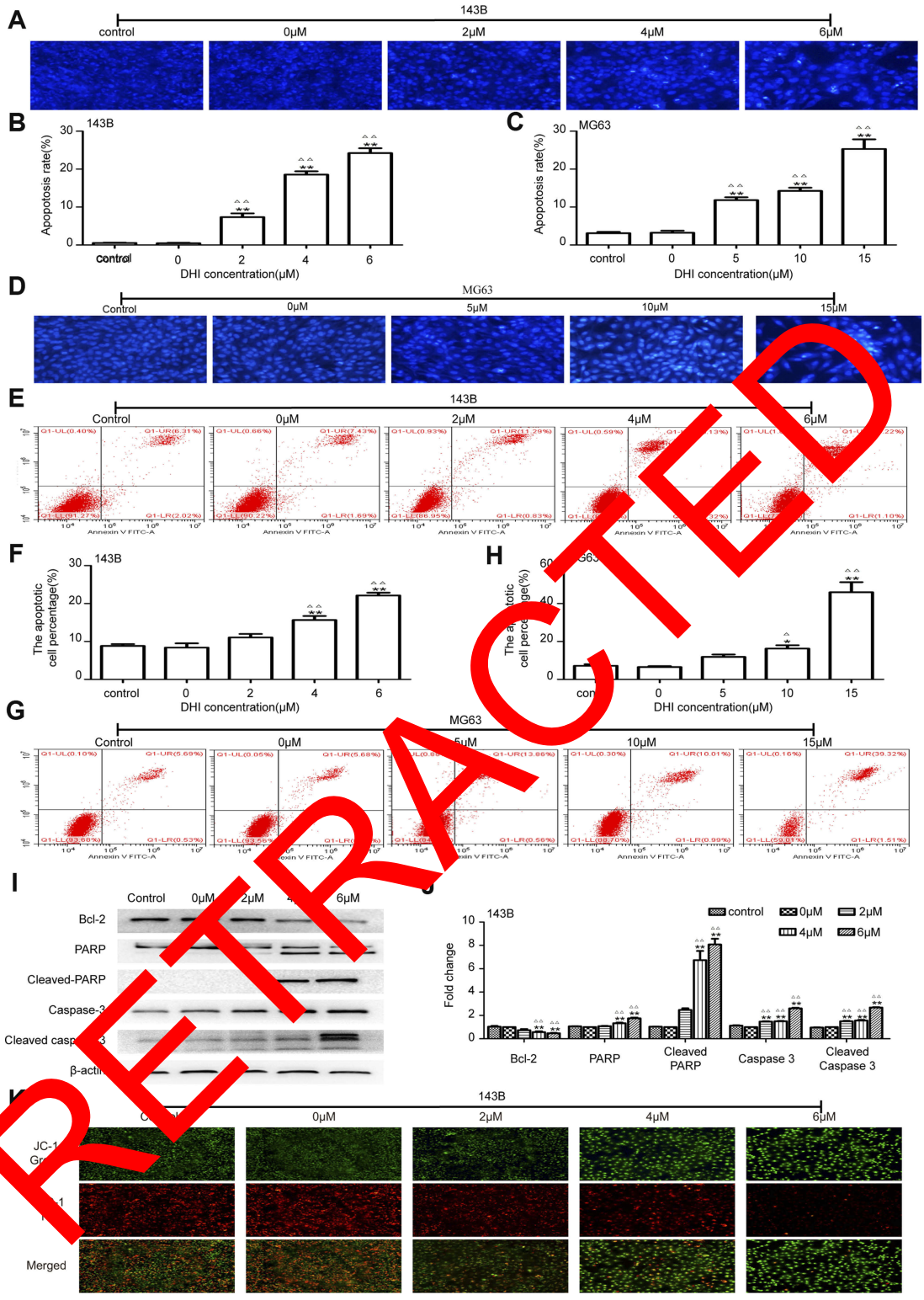

Figure 3 DHI promoted OS cell apoptosis in vitro.

Notes: (**A**–**D**) Flow cytometry was used to explore whether DHI promoted OS cell apoptosis. (**E**–**H**) Hoechst staining showed that DHI significantly promoted apoptosis of two cell lines. (**I** and **J**) Western blot analysis showing DHI downregulated Bcl-2, and upregulated PARP, Cleaved PARP, Caspase-3, and Cleaved Caspase-3. (**K**) The Δψm (red/green) alteration in 143B cells was determined by fluorescence microscopy after staining with JC-1. μM: The different concentrations of DHI; Control: Osteosarcoma 143B cells were treated with DMSO to exclude the effect of DMSO on the test. $^{\Delta}P<0.05$, vs the control group; $^{*}P<0.05$, vs 0μmol/L DHI group; $^{\Delta\Delta}P<0.01$, vs the control group; $^{**}P<0.01$, vs 0 μmol/L DHI group (n=3).

Abbreviations: OS, osteosarcoma; DHI, Dihydrotanshinone I.

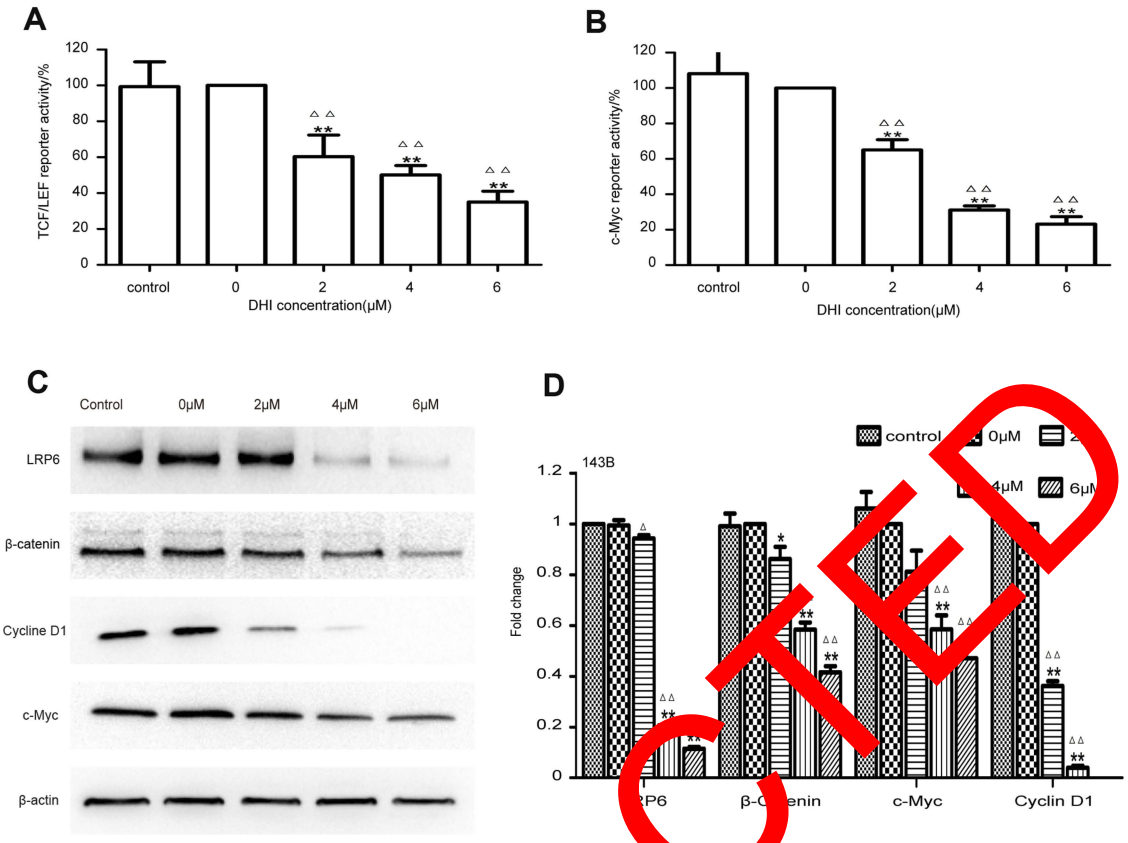

Figure 4 DHA suppresses Wnt/β-catenin signaling in human OS cells.
Notes: (**A** and **B**) The effects of DHI on the activity of the Wnt/β-Catenin signaling pathway in osteosarcoma 143B cells were detected by luciferase reporter assay. (**C** and **D**) Western blot analysis showing DHI downregulated LRP6, cyclinD1, c-MYC, and β-Catenin. The different concentrations of DHI; Control: Osteosarcoma 143B cells were treated with DMSO to exclude the effect of DMSO on the test. $^{\Delta}P<0.05$, vs the control group; *P<0.05, vs 0μmol/L DHI group; $^{\Delta\Delta}P<0.01$, vs the control group; **P<0.01, vs 0 μmol/L DHI group (n=3).
Abbreviations: OS, osteosarcoma; DHI, Dihydrotanshinone.

DHI inhibits the growth and metastasis of osteosarcoma cells in vivo

To determine whether DHI has similar effects in animals, we constructed a tumor model with 143B cells. The results showed that the tumor size was inhibited with the increase of DHI dosage (Figure 5A and B, $P<0.05$). Immunohistochemical results showed that the expression of PCNA, Bcl-2, N-Cadherin, and β-Catenin decreased (Figure 5C–J, $P<0.05$). These results suggest that DHI can inhibit the growth of osteosarcoma in vivo.

Discussion

Osteosarcoma is an invasive malignant tumor.[16] In the past, surgical resection was used as the main treatment method. With the accumulation of clinical treatment cases and the review of a large number of data, it was found that non-operative treatments, such as adjuvant chemotherapy, immunotherapy, and traditional Chinese medicine treatment, improved the survival rate of osteosarcoma patients to a certain extent,[17] but various uncontrollable factors or potential side effects make these treatments unsatisfactory.[18] Herbal and natural products have been proven to be highly suitable sources for anticancer drugs.[5–8,19] DHI is a traditional Chinese medicine extracted from *Salvia miltiorrhiza* Bge. *Salvia miltiorrhiza* Bge is mostly used to treat hematological abnormalities.[10] Recent studies have shown that DHI has anti-tumor effect in many kinds of tumors, but its mechanism has not been fully explained. It is of note, however, that the inhibitory effect of DHI on osteosarcoma in vivo and in vitro may be related to the Wnt/β-Catenin pathway.

We found that DHI could reduce the proliferation of osteosarcoma cells and the expression of Proliferating Cell Nuclear Antigen (PCNA) protein. PCNA is named for its presence in normal proliferative cells and tumor cells. Later studies have found that PCNA is closely related to cell DNA synthesis, plays an important role in the

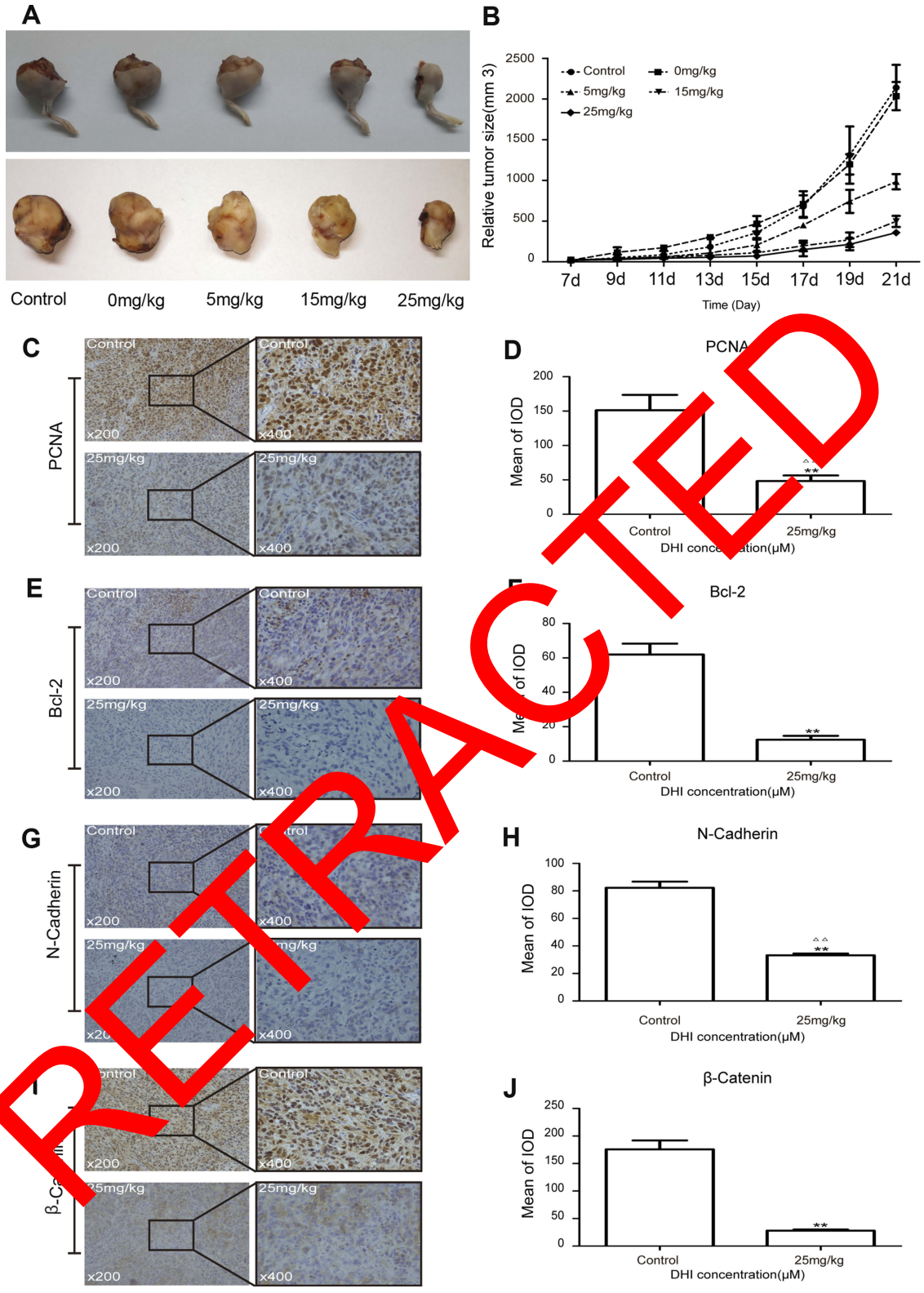

Figure 5 Effect of DHI on osteosarcoma in vivo.

Notes: (**A**) DHI treatment group showed a reduced tumor growth rate. (**B**) The DHI treatment group showed reduced tumor volumes compared with controls. (**C–J**) PCNA, Bcl-2, N-Cadherin, and β-Catenin were detected by immunohistochemistry after xenograft tumor tissues were excised. $^{\Delta\Delta}P<0.01$, vs the control group; $^{**}P<0.01$, vs 0 μmol/L DHI group (n=3).

Abbreviations: OS, osteosarcoma; DHI, Dihydrotanshinone I; IOD, integrated optical density.

initiation of cell proliferation, and is a good index to reflect the state of cell proliferation.[20]

There are three main apoptotic pathways: the death receptor (extrinsic) pathway, the mitochondrial pathway, and endoplasmic reticulum stress-mediated apoptosis.[21] The decrease of mitochondrial membrane potential is a landmark event in the early stage of apoptosis. The decrease of cell membrane potential can be easily detected through the transition of JC-1 from red fluorescence to green fluorescence. This can also be used as an early detection index of apoptosis.[22] Bcl-2, a member of the BCL2 family, is a key regulator of cell apoptosis and is known for its inhibition of mitochondrial cytochrome c release.[23] We found that DHI decreased the expression of Bcl-2 and increased the transition from red fluorescence to green fluorescence, suggesting that DHI can also induce the mitochondrial apoptosis pathway. Caspase-3 is the most important terminal shearing enzyme in the process of apoptosis. It is also an important part of the killing mechanism of CTL cells.[24] PARP is very important for the stability and survival of cells. It can be used as a marker of apoptosis and is generally regarded as a marker of Caspase-3 activation.[25] Our results suggest that DHI affects extrinsic apoptosis and the mitochondrial apoptosis pathway.

Metastasis of malignant tumors is often the main reason for the failure of tumor treatment. We found that DHI inhibited the migration and invasion of osteosarcoma and downregulated the expression of MMP-2, MMP-7, MMP-9, N-Cadherin, and snail proteins. The main function of MMP-9 is to degrade and remodel the extracellular matrix. Related reports show that the depth of invasion, metastasis distance, and vascular permeability are positively correlated with the expression level of MMP-2, MMP-7, and MMP-9.[26] Interference blocking the function of Snail can effectively inhibit tumor invasion and growth in vivo, accompanied by the increase of tumor differentiation and the marked decrease of angiogenesis and invasiveness due to MMP-9.[27] During tumor formation, the abnormal expression of N-cadherin causes the cancer cells to move from the cancer tissue to the basement membrane, adhere to and degrade the extracellular matrix, and further break through the structure of the tissue barrier, ultimately leading to local infiltration and long-distance diffusion. Related studies show that the EMT signaling pathway is activated in OS.[28] We hypothesize that DHI inhibits tumorigenesis by regulating EMT signals. To explore this possibility, we detected transcription factors related to the EMT signaling pathway, including the expression of N-cadherin and Snail. The results showed that DHI could reduce the metastasis of osteosarcoma by inhibiting EMT.

We found that DHI reduced the fluorescent expression of c-Myc and TCF/LEF fluorescent reporter plasmids (Figure 4A and B). The results suggest that DHI may regulate osteosarcoma cells through the Wnt/β-Catenin signaling pathway. In a frizzled- and disheveled-dependent manner, Wnt induces phosphorylation of its coreceptor, LRP6, and recruits the Axin-containing β-catenin destruction complex to the plasma membrane to form the signalosome. Within the signalosome, GSK3 is inhibited by phospho-LRP6, which leads to the destabilization of the β-catenin destruction complex and accumulation of β-catenin. Cumulative β-Catenin enters the nucleus and binds to the transcription factor TCF/LEF, regulating downstream target gene expression, including cyclinD1, c-myc, etc.[29–31] We found that DHI reduced the expression of LRP6, β-Catenin, cyclinD1, and c-myc, suggesting that DHI inhibited the Wnt/β-Catenin signaling pathway. The Wnt/β-Catenin signaling pathway plays an important role in regulating the biological characteristics of tumor cells and maintaining stem cell characteristics.[32–34] This pathway also regulates early and late cell apoptosis.[35] Abnormal activation of the Wnt/β-Catenin signaling pathway has been found in many tumors, including osteosarcoma.[23,30–32] In osteosarcoma cells, the Wnt/β-Catenin signaling pathway has a complex and unique regulatory effect on invasion, migration, proliferation, and differentiation. A large number of experiments have shown that inhibiting β-Catenin expression has a good anti-tumor effect. These findings also provide the basis for innovative treatment of osteosarcoma.[36,37]

DHI affects osteosarcoma not only through the Wnt signaling pathway, OS is characterized by an immature phenotype expressing primitive osteoblast markers or dysregulated signaling pathways such as hedgehog or Src pathways.[38] According to the related literature, the levels of Src-tot and pSrc416 in osteosarcoma cells are higher than those in HOb, and the expression levels of Src-tot and pSrc416 in different osteosarcoma are not the same, which leads to great differences in the resistance of osteosarcoma to different drugs.[39–41] This may partly explain the phenomenon in crystal purple. Meanwhile, most of osteosarcoma tumors exhibit p53 abnormalities.[42] According to Lauvrak,[43] 143B cells have been defined as very aggressive in terms of tumorigenicity, colony forming ability, migration/invasion, and proliferation capacity; on the contrary, SaoS2

cells have been classified as poorly aggressive. Moreover, these two cell lines possess a different p53 mutation status, being 143B p53 mutant and SaoS2 p53-null.[42] This method allows us to speculate on the possible mechanism of DHI. In our study, it was found that DHI had a stronger inhibitory effect on 143b cells than SaoS2, suggesting that DHI may upregulate the expression of p53 by activating extrinsic pathway, thus inducing p53-dependent apoptosis in human OS cells. In our further research, we will mainly analyze if DHI may inhibit osteosarcoma cell growth through p53.

Conclusion

Our results suggest that DHI can inhibit osteosarcoma in vivo and in vitro and that this effect may be related to blocking the Wnt/β-Catenin signaling pathway and activation of the apoptosis pathway. These results provide valuable experimental evidence to support DHI as a new c candidate for the prevention or treatment of osteosarcoma.

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Disclosure

The authors report no conflicts of interest [illegible] work.

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