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Proliferation, Apoptosis, and Invasion Effects of Mistletoe Alkali on Human Osteosarcoma U2OS *In Vitro*

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The objective of this study was to evaluate the effects of mistletoe alkali on human osteosarcoma cells (U2OS) *in vitro*. Osteosarcoma is the most common primary malignant bone tumor; although there are a lot of therapies such as surgery, radiotherapy, and chemotherapy, its prognosis is still very poor. There is increasing interest in the protective biological function of natural antioxidants contained in Chinese medicinal herbs, which are candidates for the prevention of tumors. Mistletoe alkali is one of the compounds extracted from *Viscum coloratum* (Komar.) Nakai, one kind of mistletoe, whose extracts contribute to the improvement of the prognosis of patients with malignancies. The effect of mistletoe alkali on the growth of U2OS cells was compared with 5-fluorouracil (5-FU), using a cell counting kit-8 (CCK-8). The influence of mistletoe alkali on U2OS's proliferation and apoptosis was tested by terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling staining and immunocytochemical staining of caspase 3 and proliferating cell nuclear antigen. Additionally, the invasion ability of U2OS cells was detected using a Boyden chamber transwell migration assay. CCK-8 assays gave an IC_{50} of 7 μg/mL for mistletoe alkali. Compared with 5-FU, mistletoe alkali inhibited U2OS proliferation and induced apoptosis more effectively. The invasion ability of U2OS was also weaker in mistletoe alkali than in 5-FU. Mistletoe alkali significantly inhibited growth and invasion abilities of U2OS cells and induced their apoptosis *in vitro*. Mistletoe alkali

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may be a more effective drug for human osteosarcoma than the standard chemotherapeutic drug 5-FU.

Key words: Mistletoe alkali – U2OS – Apoptosis – Boyden chamber transwell migration assay – Anticancer drug

Human osteosarcoma (OS) is a malignant neoplasm that originates from primitive bone-forming mesenchymal cells and produces malignant osteoid and tumor osteogenic cells. The incidence is the highest among primary malignant neoplasms. OS is the sixth most common form of childhood cancer and has a bimodal age distribution, affecting children and young adults (mostly between the age of 15 and 30 years), coincident with rapid bone growth.[1] Senior populations (age $>$ 60 years) also develop OS, but this more likely represents a secondary malignancy, frequently related to Paget's disease, medullary infarct, or prior irradiation.[1–3]

OS is a very aggressive tumor and if left untreated can easily cause distant metastasis at an early stage. This means that local therapy alone is not sufficient. Although new therapies continue to emerge, limb-salvage procedures with wide surgical margins are the mainstay of surgical intervention. However, osteosarcoma-related mortality remains high.[4,5] An effective and less cytotoxic drug is urgently needed to improve OS patients' quality of life and increase their survival rate.

Using combinations of traditional Chinese medicine and Western medicine to treat cancer patients has become more popular and widely accepted.[6,7] Because numerous anticancer agents have been derived directly from natural materials (*e.g.,* plants or microbes), it makes sense to examine the wealth of information about these materials provided by traditional Chinese medicine.[8,9]

Viscum coloratum (Komar.) Nakai[10] is a species of mistletoe that has been used as a traditional Chinese medicine and is currently used in numerous countries including the United States. Although this species contains multiple compounds of interest such as thionins, glycoprotein, and polysaccharose, we are interested in the mistletoe alkali in this study.[11] To find out whether mistletoe alkali may be beneficial in the treatment of OS, we used the OS cell line U2OS to test the effects of mistletoe alkali on cell proliferation, apoptosis, and invasion *in vitro*.

Materials and Methods

Mistletoe alkali preparation

Mistletoe alkali was prepared as described previously.[12] *Viscum coloratum* (Komar.) Nakai was immersed in an aqueous acid solution and mechanically disrupted into small particles. These particles were left to soak in the aqueous acid for 48 hours. The alkaloid was then precipitated out using an alkaline solution. The acidity aqua of mistletoe was proceeded to precipitate the different alkalinity with the aqua.

Cell lines and cell culture conditions

Human osteosarcoma cells (U2OS) (kindly provided by professor Wu Shan) were maintained in high glucose Dulbecco modified Eagle medium (H-DMEM; Nitrogen, Carlsbad, California) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah) and 1× antibiotics [100 U/mL penicillin and 100 U/mL streptomycin; all from Invitrogen (Carlsbad, California)], whereas NIH3T3 cells were cultured in H-DMEM supplemented with 15% FBS and 1× antibiotics. Both cell lines were kept at 37°C in a humidified atmosphere incubator containing 5% CO_2. To get conditioned media, we cultured 2×10^6 NIH3T3 cells in 100-mm dishes and grown in H-DMEM supplemented with 15% FBS and 1× antibiotics for 24 hours. Then the growth medium was replaced with 0.5 mL H-DMEM. After 24 hours, the growth medium was harvested and centrifuged at 1000 rpm for 3 minutes. The supernatant was filtered using a 0.2-mm filter, and flow-through was collected and kept at –20°C for further use.

Cell viability assay (Cell Counting Kit-8 assay)

Cell viability assays and growth curve analysis were performed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were seeded in a 96-well plate at a concentration of 2×10^5/mL per well. After culturing at 37°C for 24 hours, the cells were exposed to mistletoe alkali (1.25, 2.5, 5, 10, 20, 40, and 80 μg/mL) and 5-

fluorouracil (5-FU) (50, 100, 200, 400, 800, 1600, and 3200 μg/mL) for 48 hours. Nontreated cells were included as negative controls. Each condition was performed in triplicate. CCK-8 (10 μL) was added to each well, and the plate was incubated at 37°C for 1 hour. The absorbance at 450 nm was then recorded. The background absorbance reading was subtracted from these readings. The cell viability was calculated as follows: cell viability (%) = $[(OD_{experiment} - OD_{blank})/(OD_{negative\ control} - OD_{blank})] \times 100\%$, where $OD_{experiment}$ is the absorbance of a well with a treated cell and CCK-8; OD_{blank} is the absorbance of a well with medium and CCK-8 but without cells; and $OD_{negative\ control}$ is the absorbance of a well with untreated cells and CCK-8. All experiments were performed in triplicate on 3 separate occasions ($0.5 \times IC_{50}$, IC_{50}, $2 \times IC_{50}$), giving us the IC_{50} of mistletoe alkali and 5-FU.

Assessment of cell apoptosis and proliferation

The apoptotic index was determined by analyzing DNA fragmentation in the cell nuclei using the terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labeling (TUNEL) method. Specifically, we used the In situ Cell Death Detection Kit (Roche, Penzberg, Germany) following the instructions provided by the manufacturer. Cells (5×10^4/mL) were seeded in a 24-well plate. In brief, cells were cultured in complete media up to 80% confluence. The media were then gently removed, and drugs were added according to the 24-hour growth curve data. These cells were rinsed with phosphate-buffered saline (PBS) 3 times and fixed with 4% paraformaldehyde at 4°C for 30 minutes. Cell proliferation was determined by immunocytochemical (ICC) staining for proliferative cell nuclear antigen (PCNA).

Immunocytochemistry

Detection of caspase 3 and PCNA by the avidin-biotin-peroxidase (ABC) method for ICC experiments was described previously.[13] Cells (5×10^4/mL) were cultured for 24 hours, rinsed with 1× PBS 3 times, and fixed with 4% paraformaldehyde at 4°C for 30 minutes. The cells were permeabilized in 0.5% Triton ×100 on ice for 15 minutes. After blocking and quenching endogenous peroxidase activity, cells were incubated with anti-caspase 3 (1:50) and anti-PCNA (1:500) (Gene Company Ltd, Beijing, People's Republic of China) overnight at 4°C and rinsed again with 1× PBS 3 times. The presence of caspase 3 and PCNA was visualized by peroxidase staining using diaminobezidine (DAB) as substrate. Images of the antibody staining were collected under a 20× objective of an Olympus microscope with a cooled CCD camera system (both from Olympus Corporation, Tokyo, Japan).

Cell migration and invasion assays

Cell migration and invasion assays were conducted using 8-μm transwell filters (Costar, Corning, New York) with modifications as described previously.[14] For the invasion assay, the upper compartment was coated with 50 μg Matrigel (BD Bioscience, San Jose, California) to form a matrix barrier. A suspension of cells (5×10^4/mL, including nontreated, treated with different concentrations of mistletoe alkali, or 5-FU) was harvested and stained with 500 μg/mL Rodamine 123 (Invitrogen) for 15 minutes at 37°C. The cells were resuspended in new conditional media without FBS and then added to the upper compartment. The lower compartment was filled with 200 μL NIH3T3 conditioned medium to serve as a chemoattractant. The migration distances from the original location of the cells to the wells of the Boyden chambers were measured at 1, 2.5, and 4 hours, respectively. After 5 hours, the nonmigratory cells on the upper surface were removed using a cotton swab, and the cells on the lower surface were fixed. Cells that had migrated into the transwell filters were counted, and their invasion depths were measured from 10 randomly selected fields in at least 3 independent wells. The experiment was repeated 3 times independently.

Statistical analysis

All data were expressed as the mean ± SD. Differences between groups were assessed using analysis of variance (ANOVA) followed by *post hoc* Student-Newman-Keuls test and Student *t* test. $P < 0.05$ was considered statistically significant.

Results

Inhibitory effects of mistletoe alkali on proliferation of U2OS cells were detected by the CCK-8 assay

We identified the IC_{50} of mistletoe alkali and 5-FU on U2OS cells using the CCK-8 assay (Fig. 1A and 1B). When the concentration of mistletoe alkali was below 20 μg/mL, the concentration and inhibitory effects had a linear relationship. We were therefore

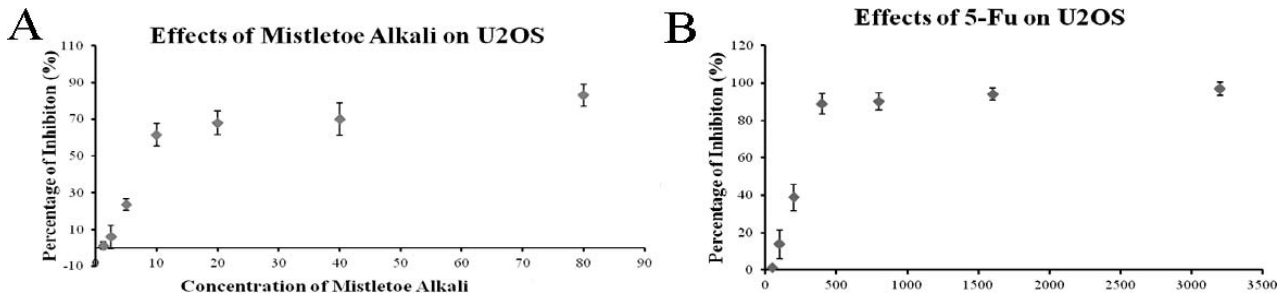

Fig. 1 Mistletoe alkali and 5-FU inhibit U2OS cell growth. Cell viability was measured by using a CCK-8 assay. U2OS cells were treated with (A) mistletoe alkali (1.25, 2.5, 5, 10, 20, 40, and 80 μg/mL) or (B) 5-FU (50, 100, 200, 400, 800, 1600, and 3200 μg/mL) for 48 hours.

able to calculate the IC_{50} of mistletoe alkali to be 7 μg/mL. Using the same method, we calculated the IC_{50} of 5-FU to be 245 μg/mL.

Mistletoe alkali induces apoptosis of U2OS cells in vitro

To quantitatively compare the apoptotic and mitotic index of U2OS cells treated with mistletoe alkali, TUNEL assays and ICC staining for PCNA and caspase 3 were performed. For PCNA and TUNEL assays, nuclei that were labeled brown were considered positive. Using Image-Pro Plus 6.0 software (Olympus), we counted 50 randomly selected cells in 5 independent areas for each condition to quantify the expression of PCNA [PCNA index (PCNALI)] and TUNEL [TUNEL index (TUNELLI)]. As shown in Fig. 2A, a trend of decreasing PCNALI was seen in the mistletoe alkali treatment group. It should also be noted that the PCNALI of the mistletoe alkali group were significantly lower than those of the 5-FU (Fig. 2Ab). These results demonstrate that mistletoe alkali inhibits cell proliferation. Additionally, there were significantly more TUNEL-positive cells in the mistletoe alkali treatment groups (7 and 14 μg/mL) than in the 5-FU positive control (Fig. 2B). This indicates that mistletoe alkali induces U2OS apoptosis *in vitro*. ICC staining using a specific antibody for the cleaved fragment of caspase 3 was in keeping with the above observations (Fig. 2C).

Mistletoe alkali inhibits migration and invasion of U2OS cells in vitro

Previous reports demonstrated that one of extracts of *Viscum coloratum* (Komar.) Nakai mistletoe lectin-I has antiproliferative and antimigration effects on various cancer cells both *in vivo* and *in vitro*.[15,16] To date, no published research has demonstrated the effect of mistletoe alkali on migration and invasion of tumor cells. To study the potential role of mistletoe alkali in migration and invasion of U2OS cells, we examined the ability of U2OS cells to invade Matrigel-coated membranes when treated with mistletoe alkali. Cells were cultured in regular media as a negative control and in 5-FU ($IC_{50,}$ 245 μg/mL) as a positive control. We measured the cell migration distances for each of the 3 treatment groups from their initial locations to the wells of the Boyden chambers at 1, 2.5, and 4 hours, respectively. As shown in Fig. 3A, cells in the mistletoe alkali treatment group migrated slowest, whereas cells in the negative control group migrated fastest ($P < 0.05$). At 5 hours, we counted the total number of the cells that had gone through the membrane (Fig. 3B). The cells treated with mistletoe alkali traversed the membrane the least, whereas those grown in negative control media did so frequently. However, compared with positive control 5-FU, the difference was not so significant.

Discussion

Human OS, a primary malignancy of the bone and the most common bone sarcoma, affects young adults and adolescents. Effective multimodal therapies including surgery, radiotherapy, and polychemotherapy have been developed, and these new treatments have significantly improved the 5-year survival rate of OS patients.[17–19] However, despite the success of chemotherapy and limb salvage (bone replacement) procedures for OS, it still has one of the lowest survival rates among pediatric cancers.[20] To improve both the survival rate and the quality of life of OS patients, novel approaches and therapies are needed. Combination chemotherapy plays an additional important role and attracts increased attention in treatment of OS.[21,22] Accordingly, identification of compounds that may increase the therapeutic index of clinical anticancer drugs be-

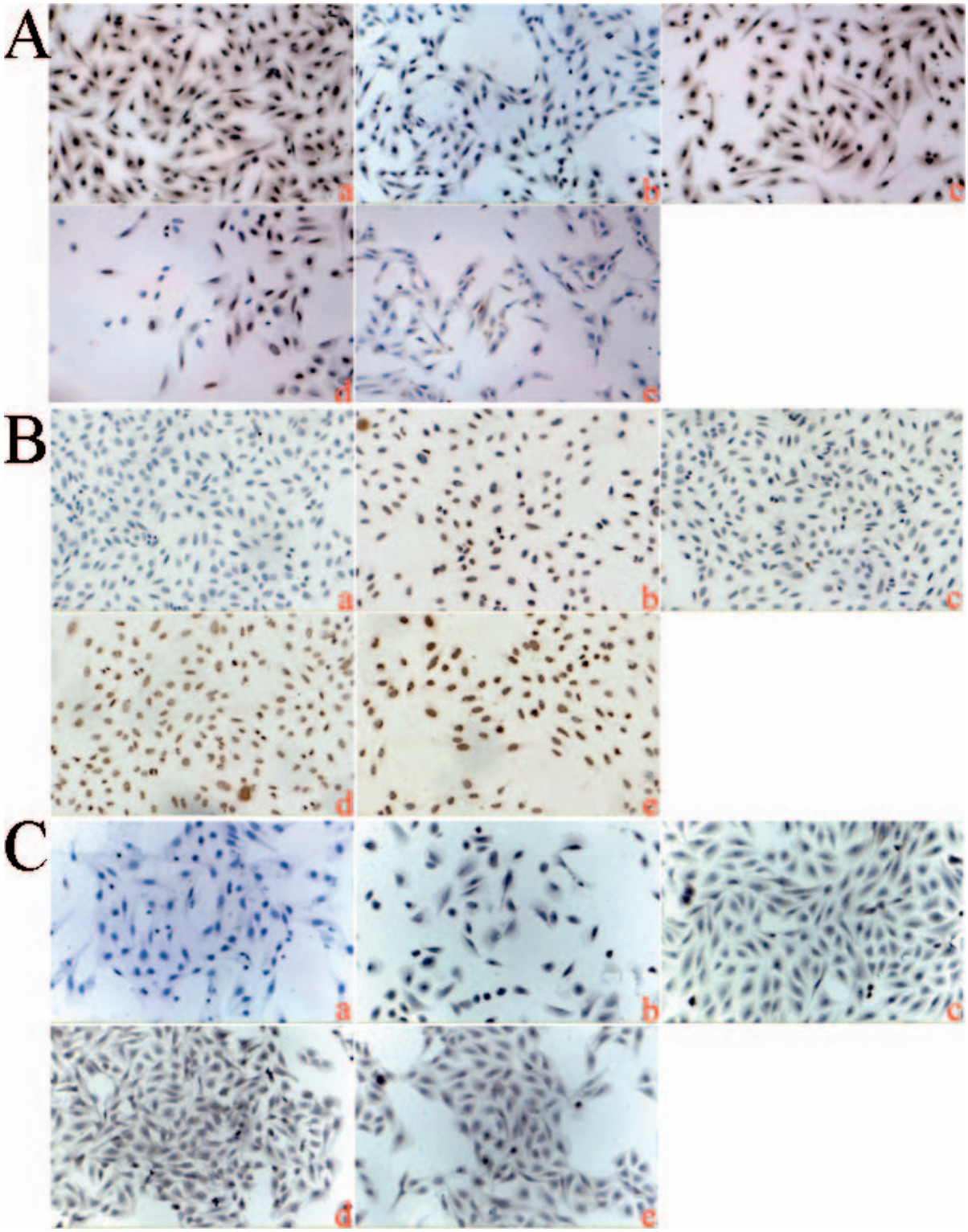

Fig. 2 Mistletoe alkali and 5-FU induce apoptosis of U2OS cells and inhibit proliferation of U2OS cells. (A) PCNA protein expression, (B) TUNEL staining, and (C) caspase 3 protein expression in U2OS cells were detected by ICC after 24-hour treatment with negative control, mistletoe alkali, or 5-FU. All pictures were taken at ×200 magnification. a, media; b, 5-FU (IC_{50}-245 μg/mL); c, mistletoe alkali-3.5 μg/mL ($0.5 \times IC_{50}$); d, mistletoe alkali-7 μg/mL (IC_{50}); e, mistletoe alkali-14 μg/mL ($2 \times IC_{50}$). Data are presented as the mean ± SD of 3 different experiments.

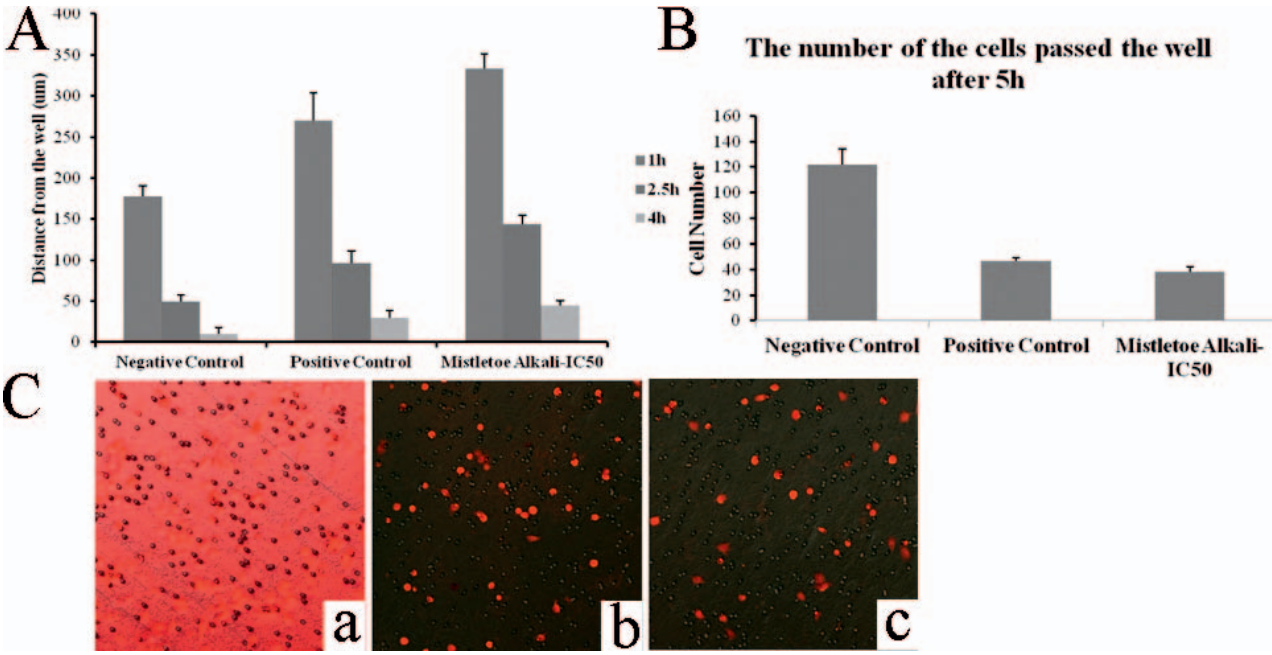

Fig. 3 Mistletoe alkali and 5-FU inhibit invasion of U2OS cells. (A) The cell migration distances for each of the 3 treatment groups, negative control, 5-FU (IC_{50}-245 μg/mL), and mistletoe alkali-7 μg/mL (IC_{50}), were measured by Boyden chambers at 1, 2.5, and 4 hours, respectively. (B) The final cell number that went through the membrane was counted at 5 hours. Invasive capacity of U2OS cells treated with mistletoe alkali was significantly decreased compared with U2OS cells in media. Negative control, media. Postive control, 5-FU (IC_{50}-245 μg/mL). a, media; b, 5-FU (IC_{50}-245 μg/mL); c, mistletoe alkali-7 μg/mL (IC_{50}).

comes more and more urgent. Therefore, naturally occurring molecules with antitumor activity and with low toxicity to normal tissues have been suggested as possible candidates for investigation of their synergistic efficacy in combination with antineoplastic drugs.[1]

Particular compounds in mistletoe belong to a group of biological response modifiers used in the treatment of cancer. Mistletoe is grown on different host trees such apple, pine, oak, fur, maple, poplar, or hawthorn.[23] In Europe, mistletoe lectin has been widely used as a complementary cancer drug for various cancers, such as gliomas,[24] acute lymphoblastic leukemia,[25] breast cancer,[26] sarcoma,[27] and others.[28] However, there has been no study showing the anticancer activity of mistletoe alkali. In this paper, we focus on the effects of mistletoe alkali on human OS cell line U2OS in terms of proliferation, apoptosis, and invasion abilities.

Although cell proliferation is essential for the health of any organism, uncontrolled growth and cell division are the distinguishing features of cancer. In our research, we used CCK8 assay to measure U2OS cell proliferation. Because the CCK-8 solution is very stable and has little cytotoxicity, incubation times of 24 to 48 hours are possible. Coupled with its higher sensitivity compared with other tetrazolium salts [*e.g.*, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT),2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), or Water Soluble Tetrazolium-1 (WST-1)], the CCK-8 reagent is ideal for our purposes. Based on our data, we calculated the IC_{50} for mistletoe alkali to be 7 μg/mL and for 5-FU to be 245 μg/mL. To understand how mistletoe alkali prevented the proliferation of U2OS cells, we performed ICC staining to test the expression of PCNA. PCNA begins to accumulate during the G1 phase of the cell cycle, is most abundant during the S phase, and declines during the G2/M phase. The temporal specificity of PCNA expression makes it an ideal marker for cell proliferation.[29] It has been reported that PCNA is highly expressed in different kinds of tumors; PCNALI is important in determining the tumor grade, recurrence span, and malignancy. The PCNALI also correlates with postoperative survival of patients.[30] Previous reports have shown that

mistletoe lectin can inhibit the proliferation of the cells (cytostatic effect), arrest the cell cycle phases, and inhibit the proliferation of several human multiple myeloma cell lines *in vitro*.[31] However, there is no relevant report of mistletoe alkali having a similar activity. In our experiments, a decreasing PCNALI was seen in the mistletoe alkali treatment group. This decrease was significantly different from the positive control, 5-FU. We could therefore infer that mistletoe alkali inhibits the proliferation of U2OS by decreasing DNA synthesis and interfering with the cell cycle.

Apoptosis is a vital physiologic process that helps to keep cell numbers in check by eliminating senescent or otherwise unuseful cells and is necessary to prevent both uncontrolled growth and tumor formation.[32] Artificially inducing apoptosis is therefore often an effective way to control cancer. Currently, a number of known anticancer drugs work by inducing apoptosis of tumor cells. 5-FU is reported to induce the apoptosis of colon carcinoma cells (HCT116), liver carcinoma cells, and breast carcinoma cells (MCF-7) and is therefore used in anticancer chemotherapy.[33] It has been previously shown that a number of different mistletoe extracts can trigger apoptosis in various cancer cell lines.[24,34,35] In this study we used both TUNEL and ICC staining of caspase 3 to detect apoptosis. TUNEL combines molecular biology and ICC and is a standard method for detecting DNA fragmentation, which is a hallmark of apoptosis. Our data show that there were significantly more TUNEL-positive cells treated with mistletoe alkali 7 (IC_{50}) and 14 μg/mL ($2 \times IC_{50}$) than in the positive control group treated with 5-FU (IC_{50}-245 μg/mL). This indicates that mistloe alkali is able to induce apoptosis more effectively than 5-FU. We also performed caspase 3 ICC staining. Caspase 3 is activated in apoptotic cells both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. Therefore, the activation of caspase-3 plays a key role and is considered the terminal event preceding apoptosis.[36–38] Using ICC staining, we found that expression of caspase-3 was highest in the cells cultured with mistletoe alkali compared with 5-FU and negative control. This supports the hypothesis that mistletoe alkali induces apoptosis of U2OS *in vitro*.

One of the most well-known characterized features of malignant tumors is their ability to invade and metastasize, eventually leading to the death of the patient. To establish distant metastases, tumor cells must cross the basement membrane, which is a thin extracellular matrix that underlies epithelia and endothelia and separates them from the stroma. In this study, we used matrigel and Boyden chambers to mimic this process. This method is considered the most reliable, reproducible, and representative of *in vivo* invasion.[39] In the assay present here, we placed the U2OS cells in the upper chamber of a Boyden migration chamber, with the upper and lower chambers separated by a membrane precoated with Matrigel. A chemoattractant (serum-free culture supernatant of NIH3T3 cells) was placed in the lower well to stimulate migration. After 5 hours, U2OS cells were recovered and counted on the lower surface of membrane. From this experiment, we found that U2OS cells cultured with mistletoe alkali were least able to invade the membrane, with fewer cells penetrating, and shallower invasion depths for those that did.

In conclusion, mistletoe alkali induces apoptosis of U2OS cells (potentially through activation of caspase 3 and decreasing DNA synthesis and interfering with the cell cycle) and clearly inhibits their ability to proliferate and invade *in vitro*. This makes it an ideal candidate for further study in relation to OS treatment.

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