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Anti-osteosarcoma effects and mechanisms of 4-*O*-amino-phenol-4′-demethylepipodophyllotoxin ether

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Abstract

The purpose of this study was to investigate the anti-osteosarcoma effects and mechanisms of 4-*O*-amino-phenol-4′-demethylepipodophyllotoxin ether (ODE), a new derivative of podophyllotoxin. The results showed that ODE inhibited proliferation of K562, OS-9901, CNE, BGC-823 and Tca-8113 cells in a time- and concentration-dependent manner as determined by microculture tetrazolium (MTT) assay. OS-9901 and K562 cells treated with ODE for 24 h showed cell cycle arrest at G_2/M and a parallel decrease in G_0/G_1 and S phase as detected by flow cytometry (FCM). Meanwhile, a fraction of cells with hypodiploid DNA content representing apoptosis were detected by FCM. Morphology observation also revealed typical apoptotic features, including shrinkage of cellular and nuclear membranes, condensed heterochromatin around the nuclear periphery and cytoplasmic vacuolation in OS-9901 cells. Under a confocal laser scanning microscope, intracellular Ca^{2+} and Mg^{2+} concentrations were greatly increased whereas the pH value, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) were markedly reduced in OS-9901 cells after treatment with ODE. Taken together, these results suggest that the anti-osteosarcoma mechanisms of ODE are attributed to apoptosis through increasing intracellular Ca^{2+} and Mg^{2+} concentrations, and reducing pH value, MMP and ROS.

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Introduction

Osteosarcoma is the most common primary malignant bone tumour in children and adolescents (Link & Eilber 1989). It is a highly aggressive neoplasm typically composed of spindle cells producing osteoid. Amputation and limb-sparing surgery are the main treatments but the two-year overall survival rate is only 15 – 20%. The outcome of osteosarcoma has not improved significantly over the last several decades (Weinfeld & Dudley 1962; Marcove et al 1970; Friedman & Carter 1972), because approximately 15 – 20% of patients present with radiographically detectable metastases (Link & Eilber 1989; Meyers & Gorlick 1997; Kaste et al 1999), about 80% of patients with localized osteosarcoma develop metastatic disease following surgical resection (Weinfeld & Dudley 1962; Dahlin & Coventry 1967; Marcove et al 1970; Friedman & Carter 1972), and virtually all patients are presumed to have subclinical, microscopic metastases (Link et al 1986, 1991). Death from osteosarcoma is usually the result of progressive pulmonary metastasis with respiratory failure due to widespread disease (Link & Eilber 1989; Meyers & Gorlick 1997; Rosen et al 2000). Thus, the successful treatment of osteosarcoma requires the use of systemic chemotherapy, and the effective chemotherapy for patients with osteosarcoma has led to significant improvement in prognosis with a 5-year disease-free survival of approximately 70% (Meyers et al 1992; Meyers & Gorlick 1997; Provisor et al 1997). The current protocol for the treatment of osteosarcoma is a combination of surgery and chemotherapy. A regimen of multiple drugs, such as the combination of adriamycin and high-dose methotrexate with vincristine, is now

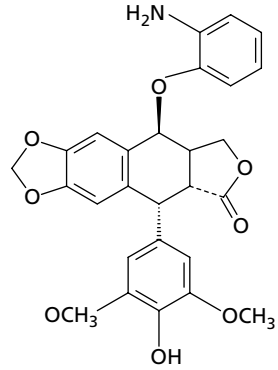

Figure 1 Chemical structure of ODE.

considered as an essential treatment for osteosarcoma (DeVita et al 1997; Ferguson & Goorin 2001). Recently, many reports suggest that the combination of ifosfamide and etoposide has significant activity and might improve the outcome for osteosarcoma patients with poor histological responses (Harris et al 1995, 1998; Goorin et al 2002). Unfortunately, the use of multi-agent treatments for osteosarcoma is associated with acute and long-term toxicity and their clinical effectiveness has always been restricted. Thus, it appears that we have reached the limit in the survival of osteosarcoma patients achievable with currently available chemotherapy. Further improvements in outcome of osteosarcoma will depend on the discovery of new drugs that possess potent activity and lower toxicity.

Etoposide, a semisynthetic derivative of podophyllotoxin, is widely used as an antineoplastic agent and is useful in the clinical treatment of several types of neoplasms, including testicular cancer, small-cell lung cancer, Hodgkin and non-Hodgkin or other lymphomas, leukaemia, Wilm's tumour, Kaposi's sarcoma and so on (Yalamati & William 1998; Gordaliza et al 2004). More recently, it has been considered as a promising chemotherapeutic agent and might improve the outcome for osteosarcoma patients with poor histological responses. Nonetheless, its clinical effectiveness is restricted due to several limitations, including myelosuppression, drug resistance, cytotoxicity towards normal cells and inactivation caused by metabolism. To overcome the limitations of these compounds and to develop new compounds with stronger antineoplastic activity and lower toxicity, a number of derivatives of podophyllotoxin have been obtained through performing structural modifications. This experiment was designed to investigate the anti-osteosarcoma effects and mechanisms of 4-*O*-amino-phenol-4′-demethylepipodophyllotoxin ether (ODE), a new derivative of podophyllotoxin (Figure 1).

Materials and Methods

Drugs and chemicals

4-*O*-Amino-phenol-4′-demethylepipodophyllotoxin ether (ODE), purity > 99%, a generous gift from the State Key Laboratory of Applied Organic Chemistry, Lanzhou University (Lanzhou, China), was dissolved in 5% dimethyl sulfoxide (DMSO). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St Louis, MO). RPMI-1640 was obtained from GIBCO BRL (Grand Island, NY). Newborn calf serum was purchased from Hangzhou Si-Ji-Qing Biotechnology Co. (Hangzhou, China). Fluo-3/AM, Mag-Fluo-4/AM, Carboxy SNARF-1/AM, Mito Tracker Green FM and 2′,7′-difluorofluorescein diacetate were purchased from Molecular Probes Inc. (Eugene, OR). Other chemicals were of analytical purity.

Cell culture and MTT assay

Human osteosarcoma 9901 (OS-9901) cell line was provided by the Department of Orthopedics, Tangdu Hospital, Fourth Military Medical University (Xi'an, China). Human chronic myelogenous leukaemia K562, low differential human gastric cancer line BGC-823, human nasopharyngeal carcinoma CNE and human tongue scale cancer Tca-8113 cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in complete RPMI-1640 medium containing 10% heat-inactivated newborn calf serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μ g mL^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO_2, and routinely passaged for 2–3 days.

Cytotoxicity was measured by microculture tetrazolium (MTT) assay (Mosmann 1983) with slight modification. Briefly, exponentially growing cells were washed and resuspended in complete RPMI-1640 medium to a density of 1×10^5 cells mL^{-1}. Samples (100 μL) of cells containing ODE were seeded in quadruplicate into a 96-well flat bottom microculture plate (Costar, Corning, USA) for varying periods of time. At the end of the incubation period, MTT (5 mg mL^{-1}, dissolved in PBS) 10 μL was added to each well and further cultured for another 4 h, then SDS 100 μL (10%, w/v, dissolved in 0.01 M HCl) was added and mixed thoroughly to dissolve formazan crystals at 37°C. After shaking plates for 10 min, optical density (OD) was measured at 570 nm with a Microplate Reader (ELx800 Instruments, BioTEK, USA).

Morphological features of apoptosis

Observation by inverted microscopy

OS-9901 cells (1×10^6 cells mL^{-1}) were grown in medium containing 2.5~200 mg L^{-1} ODE for 24 h, then stained with Wright–Giemsa and observed under an inverted microscope (Olympus, Japan).

Observation by transmission electron microscopy

OS-9901 cells treated with ODE 22.2 mg L^{-1} for 24 h were prefixed in 2% cacodylate-buffered glutaraldehyde, post-fixed in 1% osmium tetraoxide, dehydrated in graded series of alcohol and embedded in Epon (PolyBed 812). Sections were stained with uranyl acetate and lead citrate and were examined with an EM-1230 electron microscope (Japan).

Cell cycle analysis by flow cytometry

Treated K562 and OS-9901 cells, as described above, were washed in PBS by centrifugation, and fixed in ice-cold 70% ethanol at −20°C for at least 24 h. The cells after fixation were washed in PBS (without Ca^{2+} and Mg^{2+}) and stained with propidium iodide (PI) solution containing PI 50 mg L^{-1} and RNase 50 mg L^{-1} for 30 min at room temperature in the dark. The sample was read on a Coulter Epics XL flow cytometry (Beckman-Coulter Inc, Fullerton, CA). The distribution of cell cycle was calculated using Multicycle software (Phoenix Flow System, San Diego, CA).

Determination of intracellular Ca^{2+} and Mg^{2+} concentration, pH value, mitochondrial membrane potential and reactive oxygen species

To measure intracellular Ca^{2+} and Mg^{2+} concentration, pH value, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS), Fluo-3/AM, Mag-fluo-4, Carboxy SNARF-1/AM, Mito Tracker Green FM and 2′, 7′-difluorofluorescein diacetate were used respectively. After treatment as described above, OS-9901 cells were harvested by trypsinization and washed twice in RPMI-1640 medium, and then loaded with Fluo-3/AM (5 μM), Mag-fluo-4 (5 mM), Carboxy SNARF-1/AM (10 μM), Mito Tracker Green FM (1.25 μM) and 2′,7′-difluorofluorescein diacetate (1.25 μM), respectively, at 37°C for 30–45 min according to the manufacturer's instructions. The cells were again washed twice in RPMI-1640 medium. The fluorescence intensity changes of intracellular Ca^{2+}, Mg^{2+}, pH value, MMP and ROS were measured using confocal laser scanning microscopy (Leica TCS SP2, Leica Microsystems Heidelberg GmbH, Mannheim, Germany).

Statistical analysis

Data were expressed as mean ± s.d. Statistical analysis was made using analysis of variance followed by Student–Newman–Keuls' post-hoc test for multiple comparisons with the computer statistical package SPSS 12.0 (for Windows). $P < 0.05$ was considered statistically significant.

Results

Inhibitory effect on cell proliferation

To verify the antineoplastic effect of ODE in-vitro, cytotoxicity was measured by MTT assay. ODE significantly inhibited proliferation of K562, OS-9901, CNE, BGC-823 and Tca-8113 cells in a time- and concentration-dependent manner (Figure 2). The IC50 values were 0.56, 25.5, 19.02, 66.03 and 33.5 mg L^{-1} when treated with ODE for 72 h, respectively. The inhibitory effect of ODE on cell proliferation is general for different cell lines, but the inhibitory effect on K562 cell is the most potent. Among adherent cells, 200 mg L^{-1} of ODE had the highest inhibition rate on OS-9901 cells.

Cell cycle analysis and evaluation of apoptotic cell death

Flow cytometric studies of the DNA content of ODE-treated K562 and OS-9901 cells and their untreated counterparts were performed to assess the effect of ODE on the cell cycle progression. Figures 3 and 4 show the flow cytometric analysis of the cell cycle distribution of K562 and OS-9901 cells exposed to different concentrations of ODE (6.25–25 mg L^{-1} for K562 cells and 7.5–66.7 mg L^{-1} for OS-9901cells) for 24 h. The OS-9901 cells of the control group showed a DNA content distribution representative of an asynchronous, exponentially growing cell population. Exposure to ODE induced a concentration-dependent accumulation of cells in G_2 phase with a corresponding decrease in G_1 and S phase. The cell cycle distribution of K562 cells induced by ODE was similar to OS-9901 cells.

Meanwhile, a fraction of cells with hypodiploid DNA content representing apoptosis was detected. ODE concentration-dependently increased the percentage of sub-G_1 DNA content in K562 and OS-9901 cells, thus supporting the hypothesis of an apoptotic mode for cell death (Figures 3 and 4).

Morphological features of apoptosis

Typical apoptosis characteristics were present in the OS-9901 cells treated with ODE for 24 h. Nuclear condensation, chromosome fragmentation and apoptotic bodies were observed by inverted microscopy (data not shown). Electron microscopic observation also revealed typical apoptotic features, including shrinkage of cellular and nuclear membranes, condensed heterochromatin around the nuclear periphery and cytoplasmic vacuolation in the OS-9901 cells treated with ODE 22.2 mg L^{-1} for 24 h (Figure 5).

Effect of ODE on intracellular Ca^{2+} and Mg^{2+} concentrations

Fluo 3-AM is an acetoxymethyl ester derivative of Fluo 3 that can be easily loaded into cells by incubation. Then it is hydrolysed to fluo-3 by intracellular lipase. Fluo 3 is practically non-fluorescent in its free ligand form, but its fluorescence increases 60∼80 times when it forms complexes with calcium. The changes in fluorescent intensity might represent the corresponding alteration of Ca^{2+} concentration. Thus, it has been widely used with confocal laser fluorescent microscopy because the microscope has an argon laser. Like Fluo-3, Mag-fluo-4, used as an intracellular Mg^{2+} indicator, is essentially non-fluorescent in the absence of divalent cations and exhibits strong fluorescence enhancement with no spectral shift upon binding to Mg^{2+}. To investigate the mechanism of antineoplastic effects of ODE on OS-9901 cells, we examined the changes of intracellular Ca^{2+} and Mg^{2+} concentrations with molecular probes as detected by confocal laser scanning microscopy for 24 h. As shown in Figure 6, the fluorescence intensity of intracellular Ca^{2+} was significantly increased in a concentration-dependent manner after treatment with ODE when compared with untreated cells. Meanwhile, the changes of Mg^{2+} fluorescence intensity were also significantly augmented after treatment with ODE of 2.5–200 mg L^{-1}.

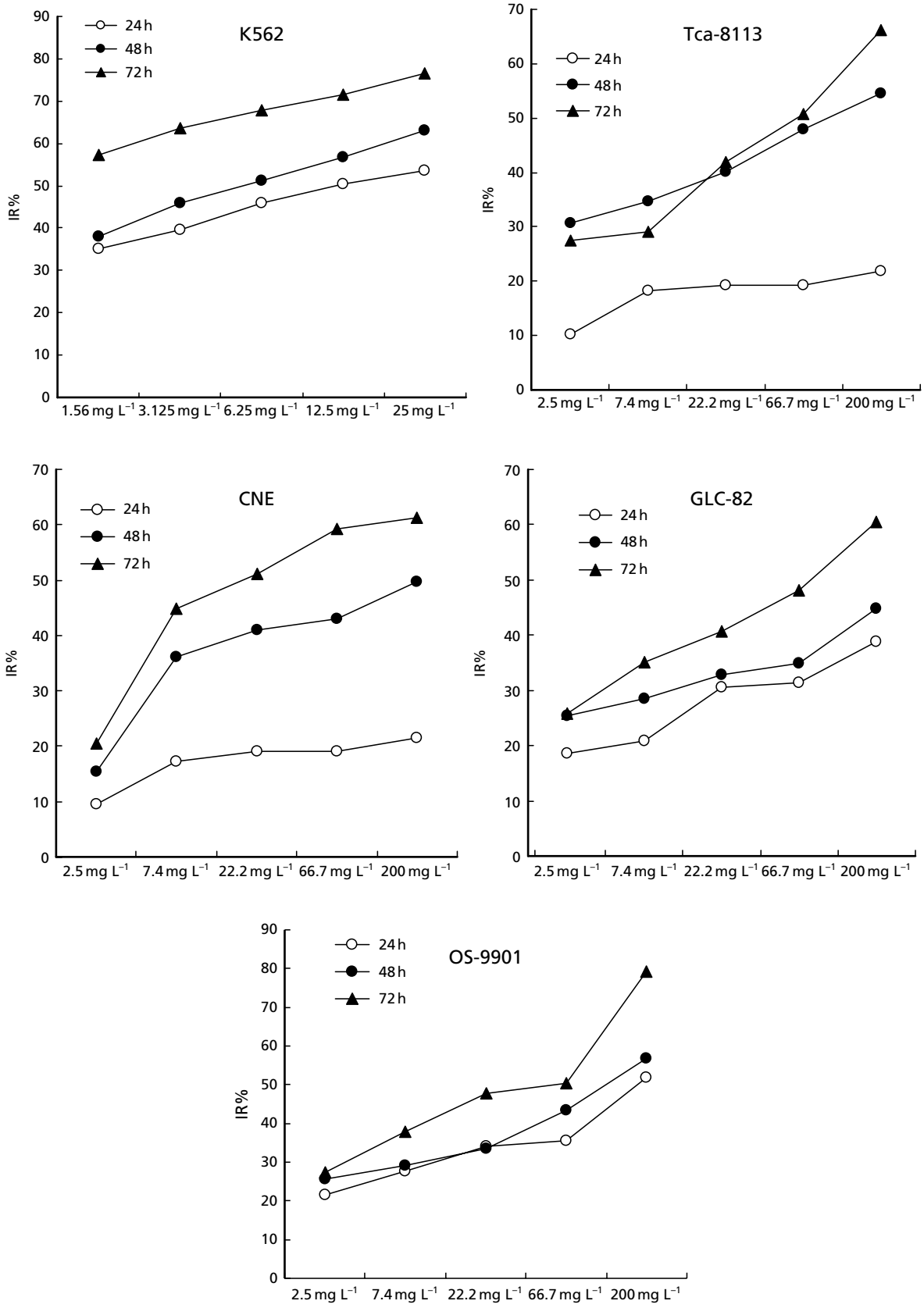

Figure 2 Inhibitory effects of ODE on cancer cells after 24, 48 and 72 h treatment as measured by MTT assay. Inhibition rate (IR%) = $(1 - OD570_{Treated}/OD570_{Control}) \times 100$.

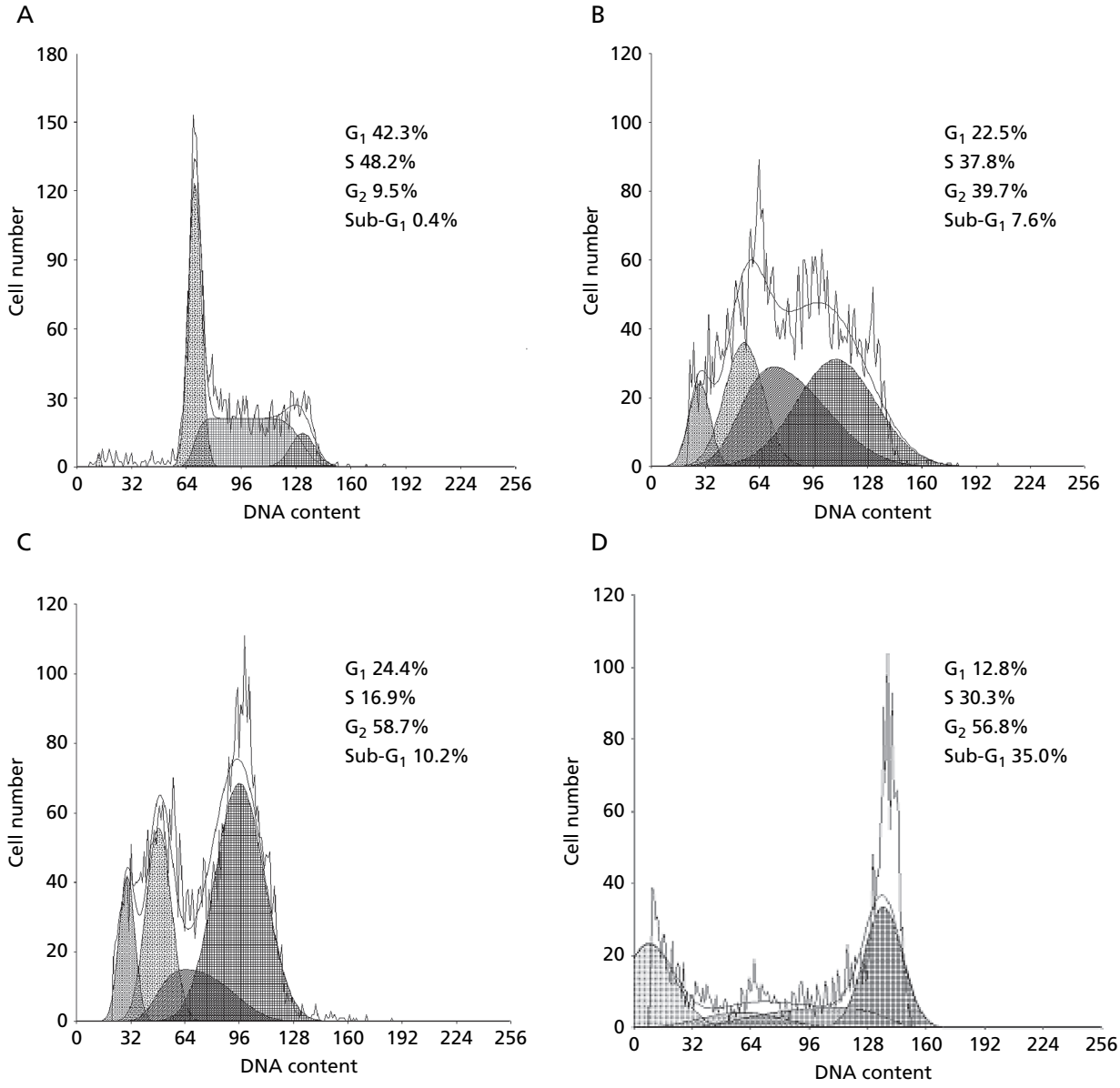

Figure 3 Effect of ODE on cell cycle distribution and apoptotic index of OS-9901 cells as determined by flow cytometry. Control cells (A); OS-9901 cells treated with ODE 7.5 mg L^{-1} (B); OS-9901 cells treated with ODE 22.2 mg L^{-1} (C); OS-9901 cells treated with ODE 66.7 mg L^{-1} (D). Sub-G_1 peak was found before G_1 phase, which was a typical apoptotic cell peak. Apoptosis rate was calculated using Multicycle software.

Effect of ODE on intracellular pH value

Carboxy-SNARF-1 is the most useful fluorescent indicator for determining the distribution of hydrogen ions in cultured cells and is widely used for monitoring intracellular pH. Intracellular acidification has been suggested to play a pivotal role in the process of apoptosis. We examined the changes of intracellular pH value induced by ODE for 24 h using confocal laser scanning microscopy. As with intracellular Ca^{2+} and Mg^{2+} changes, the fluorescence intensity of intracellular H^+ was significantly increased in a concentration-dependent manner after treatment with ODE as compared with untreated cells (Figure 7). This result indicates that ODE concentration-dependently reduced intracellular pH value.

Effect of ODE on intracellular mitochondrial membrane potential

Mito Tracker Green FM is a dye used for staining mitochondria, which is taken up into the mitochondrial membrane in a membrane-potential-sensitive manner. It is essentially non-fluorescent in aqueous solutions, only becoming fluorescent once it accumulates in the lipid environment of mitochondria. Mitochondria play an important role in apoptosis induction under both physiological and pathological conditions. Monitoring the mitochondrial membrane potential (MMP) using a fluorescent technique has generally been adopted as an indicator of cell apoptosis. To investigate the mechanism of the antineoplastic effect of ODE

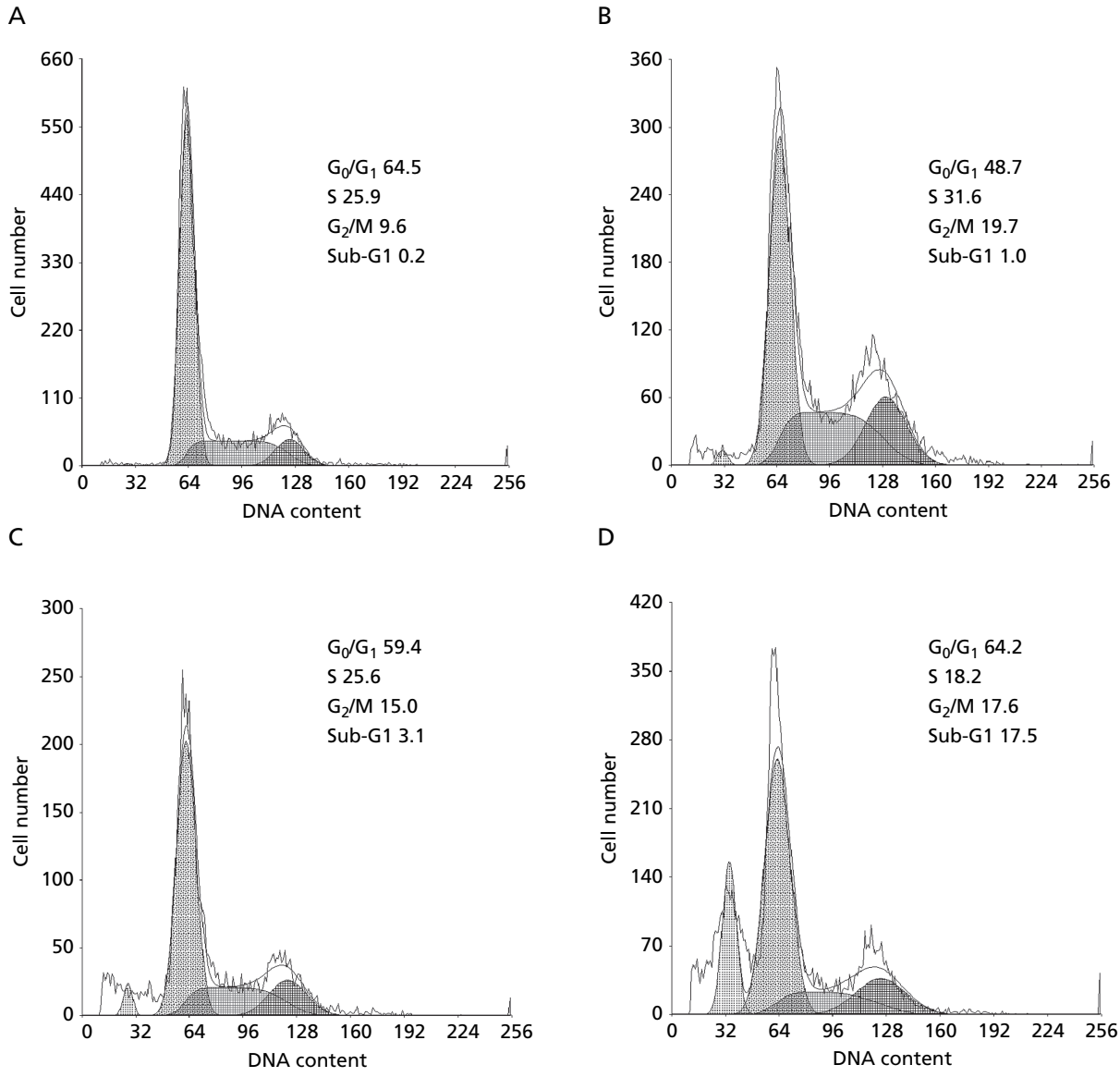

Figure 4 Effect of ODE on cell cycle distribution and apoptotic index of K562 cells as determined by flow cytometry. Control cells (A); K562 cells treated with ODE 6.25 mg L^{-1} (B); K562 cells treated with ODE 12.5 mg L^{-1} (C); K562 cells treated with ODE 25 mg L^{-1} (D). Sub-G_1 peak was found before G_1 phase, which was a typical apoptotic cell peak. Apoptosis rate was calculated using Multicycle software.

on OS-9901 cells, we examined the fluorescence intensity changes of MMP with confocal laser scanning microscopy. As shown in Figure 8, ODE 66.7–200 mg L^{-1} reduced MMP in a concentration-dependent manner as compared with control cells.

Effect of ODE on intracellular reactive oxygen species (ROS)

Production of ROS was measured in OS-9901 cells by confocal laser scanning microscopy using 2′,7′-difluorofluorescein diacetate as a probe. Intracellularly, the diacetate derivative is cleaved by endogenous esterases, releasing the corresponding non-fluorescent dichlorodihydrofluorescein derivative. This is readily oxidized back to fluorescein by ROS, thereby acting as a fluorogenic probe to detect oxidative activity in cells and tissues. As shown in Figure 9, intracellular fluorescence intensity of ROS was markedly reduced by ODE 66.7 and 200 mg L^{-1} as compared with the control group.

Discussion

Osteosarcoma is the most common and highly aggressive primary malignant bone tumour in children and adolescents (Link & Eilber 1989). It is confirmed that adjuvant chemotherapy produces higher disease-free survival

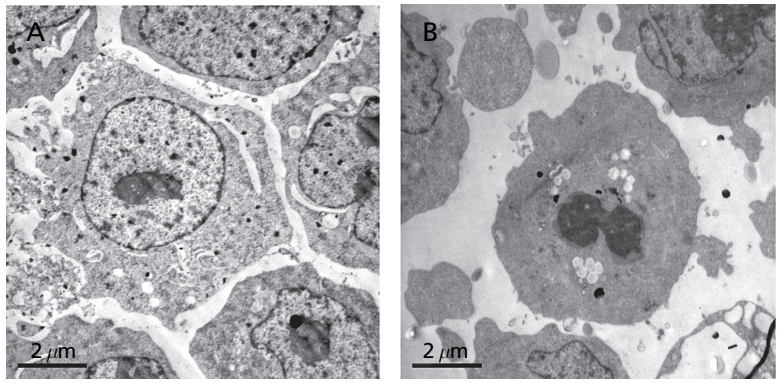

Figure 5 Morphological changes of apoptosis under transmission electron microscope in OS-9901 cells treated for 24 h. Untreated OS-9901 cells (A); OS-9901 cells treated with ODE 22.2 mg L^{-1} (B). After 24 h treatment, OS-9901 cells were prefixed in 2% cacodylate-buffered glutaraldehyde, post-fixed in 1% osmium tetraoxide, dehydrated in graded series of alcohol and embedded in Epon (PolyBed 812). Sections were stained with uranyl acetate and lead citrate.

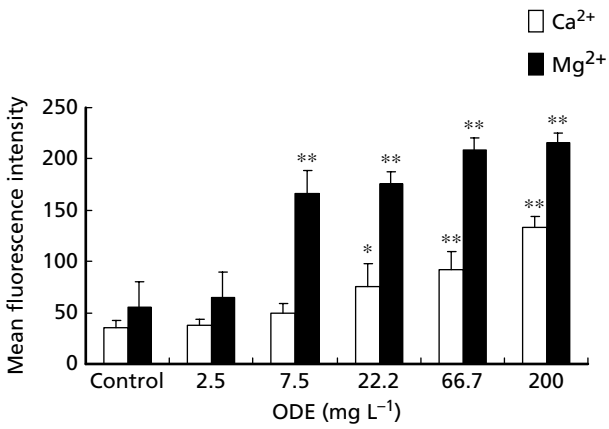

Figure 6 Effect of ODE on the fluorescence intensity of intracellular Ca^{2+} and Mg^{2+} concentrations detected by confocal laser scanning microscopy in OS-9901 cells treated for 24 h. After 24 h treatment, OS-9901 cells were loaded with Fluo-3/AM (5 μM) and Mag-fluo-4 (5 mM) for 45 min at 37°C. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group (analysis of variance).

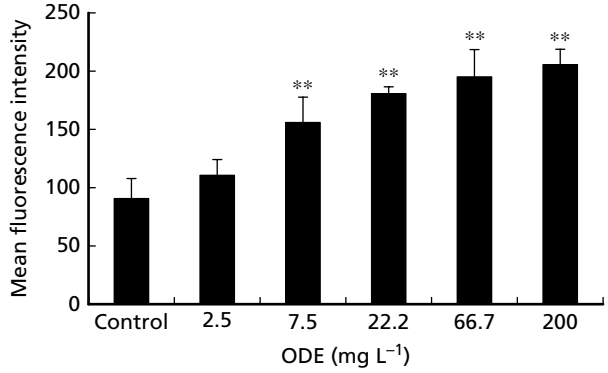

Figure 7 Effect of ODE on the fluorescence intensity of intracellular H^{+} concentration detected by confocal laser scanning microscopy in OS-9901 cells treated for 24 h. After 24 h treatment, OS-9901 cells were loaded with Carboxy SNARF-1/AM (10 μM) for 45 min at 37°C. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group (analysis of variance).

rates for patients with osteosarcoma (Eilber et al 1987). The most effective chemotherapeutic agents currently in use include high-dose methotrexate, doxorubicin, cisplatin and ifosfamide/etoposide. The optimal schedule of therapy is still being investigated, as is the role of dose intensification. Unfortunately, some groups of patients whose tumours show relatively low degrees of necrosis after administration of chemotherapy remain at high risk of eventual relapse, and some patients who present with overt metastatic disease continue to have an unsatisfactory outcome. These groups, in particular, may benefit from future investigations into novel agents. More recent studies show that etoposide, a derivative of podophyllotoxin, is a promising chemotherapeutic agent and might improve the outcome for osteosarcoma patients with poor histological responses (Goorin et al 2002). Podophyllotoxin, a traditional antineoplastic drug, raised promise for investigating more effective anti-osteosarcoma agents. This study demonstrated that ODE, a novel podophyllotoxin derivative, exerted general cytotoxic effects on OS-9901, K562, BGC-823, CNE and Tca-8113 cells in a concentration- and time-dependent manner. Among adherent cell, 200 mg L^{-1} of ODE had the highest inhibition rate on OS-9901 cells. Meanwhile, cell cycle distribution was also performed in OS-9901 and K562 cells to evaluate whether ODE was able to affect cell progression through any particular phase of the division cycle. Our results indicated that ODE dose-dependently accumulated OS-9901 and K562 cells in G_2 phase of the cell cycle and with a concurrent decrease in S and G_1 phase. This suggests that the ODE-induced antineoplastic effect may be associated with both cell cycle progression regulatory mechanisms and the triggering of cell death. The presence of a fraction of cells with hypodiploid DNA content after 24-h exposure to ODE in OS-9901 and K562 suggests the triggering of cell death may be via apoptotic mechanism. This is supported by the ODE-induced cell morphological

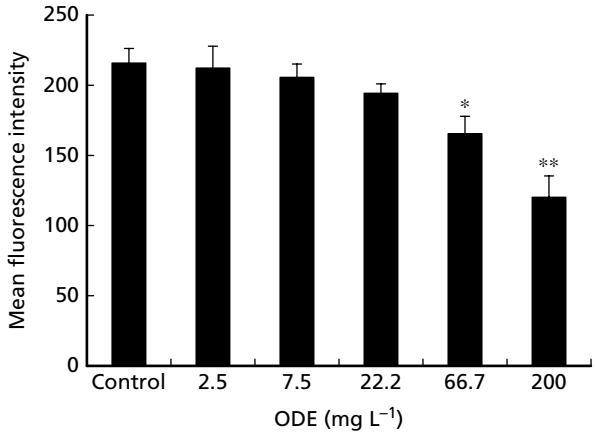

Figure 8 Effect of ODE on the fluorescence intensity of mitochondrial membrane potential (MMP) detected by confocal laser scanning microscopy in OS-9901 cells treated for 24 h. After 24 h treatment, OS-9901 cells were loaded with Mito Tracker Green FM (1.25 μM) for 45 min at 37°C. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group (analysis of variance).

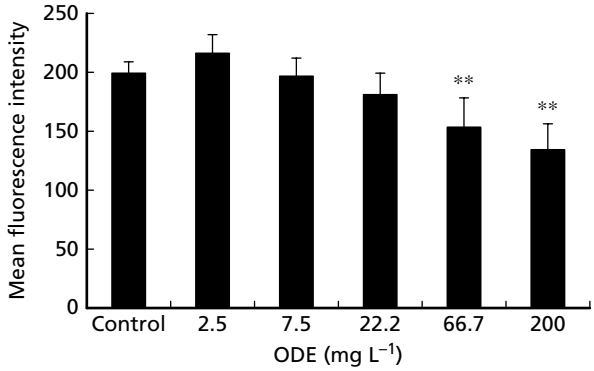

Figure 9 Effect of ODE on the fluorescence intensity of reactive oxygen species (ROS) detected by confocal laser scanning microscopy in OS-9901 cells treated for 24 h. After 24 h treatment, OS-9901 cells were loaded with 2′,7′-difluorofluorescein diacetate (1.25 μM) for 45 min at 37°C. $^{**}P < 0.01$ vs control group (analysis of variance).

features showing typical apoptosis characteristics in OS-9901 cells treated with ODE for 24 h. Due to the apotosis-induced mechanism, the inhibitory rate of ODE 200 mg L^{-1} on cancer cells for 72 h did not exceed 80%.

Induction of apoptosis and inhibition of cell proliferation are considered important cellular events that can account for the antineoplastic effects of podophyllotoxin derivatives such as etoposide (Than et al 2001). Apoptosis is not only a genetically controlled mechanism essential for the maintenance of tissue homoeostasis, proper development and elimination of unwanted cells such as cancer cells (Wyllie et al 1980), but also a commonly accepted mechanism of antineoplastic effect for chemotherapeutic agents (Silva et al 1996). Besides inhibition of cancer cell proliferation, ODE also induced typical apoptosis characteristics as detected by flow cytometry and morphological methods in OS-9901 or K562 cells. It is presumed that perturbations in intracellular ion homoeostasis could be a conspicuous manifestation of apoptosis, due to the multitude of proteins that are activated in the apoptotic cascade, and which invariably depend on the existence of certain intracellular ions (McConkey et al 1989; Cotter & Fernandes 1993; McConkey & Orrenius 1996; Zhang et al 2005). Particular emphasis has been placed on the influence of Ca^{2+} ions because Ca^{2+} is one of the most important intracellular messengers. Intracellular Ca^{2+} plays a central role in the induction of apoptosis. An increase in cytosolic Ca^{2+} concentration has often been linked to the activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease responsible for cleavage of DNA linker regions to generate nucleosomal (180–200 bp) fragments during the degradation phase of apoptosis cell death. More recently, the influence of Mg^{2+} ions on apoptosis has also received some attention because intracellular Mg^{2+} is the most abundant divalent metal ion in eukaryotic cells, and the role of Ca^{2+} as an intracellular messenger is incomplete without the coexistence of internal Mg^{2+} ions. Mg^{2+} is thought to be required in excess for Ca^{2+} to elicit a response. The regulatory function of Ca^{2+} is carried out in synergy with the structural function of Mg^{2+}. The Ca^{2+}/Mg^{2+} binding sites are occupied by Mg^{2+} in-vivo to stabilize the structure of proteins, while the Ca^{2+}-specific sites perform the regulatory functions. The sole contribution of Mg^{2+} to apoptosis has been attributed to its absolute requirement in the initial breakdown of DNA into large fragments of 300 and 50 kbp, before the dispensable event of internucleosomal cleavage (Cain et al 1994; Zhang et al 2005). In this experiment, ODE elevated intracellular Ca^{2+} and Mg^{2+} concentrations in OS-9901 cells. This indicates that Mg^{2+} may be an adjunct to Ca^{2+} ions responsible for both endonuclease activity and apoptosis induction. In addition, the changes in intracellular ions such as $Ca^{2+}$and Mg^{2+} may induce mitochondrial apoptosis and reduce membrane potential, and monitoring the mitochondrial membrane potential using fluorescent technique has generally been adopted as an indicator of cell apoptosis (Nicholls & Ward 2000; Bolduc et al 2004; Zhang et al 2005). In our study, ODE also led to the decrease of mitochondrial membrane potential, accounting for the apoptotic phenomenon of tumour cells.

Moreover, intracellular acidification was suggested to play a pivotal role in the process of apoptosis, either by directly triggering the apoptosis pathway or by providing a permissive milieu for the enzymatic processes that control programmed cell death (Gottlieb et al 1996; Hirpara et al 2001). In our work, ODE strongly decreased intracellular pH value in OS-9901 cells as compared with the control group. This suggests the possibility that the reduction in intracellular pH value is associated with apoptosis, which may contribute to the antineoplastic effect of ODE.

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2), are produced exogenously by ionizing radiation or specific chemical agents and endogenously as a result of oxygen metabolism. Many diseases are linked directly or indirectly to ROS processes, including cancer induction, development and propagation (Halliwell & Gutteridge 1999). Accumulating data have demonstrated that ROS and mitochondria play an

important role in apoptosis induction under both physiological and pathological conditions. Interestingly, mitochondria are both a source and target of ROS. Cytochrome *c* release from mitochondria, which triggers caspase activation, appears to be largely mediated by direct or indirect ROS action. On the other hand, recent evidence has added a newer dimension to the effects of elevated intracellular ROS by demonstrating that a pro-oxidant state amplifies cell proliferation or anti-apoptosis, either via direct stimulation of cell division and activation of transcription or indirectly by inhibiting the execution of cell death signal. For example, ascorbic acid, as a potent antioxidant, may scavenge ROS and then block drug-mediated apoptosis induction allowing cancer cells to become insensitive to chemotherapeutics (Wenzel et al 2004). These findings seem to tie up well with the observation that certain cell types, in particular cancer cells, constitutively generate ROS that function as autocrine growth stimulation signals and inhibit the execution of the death signal. Therefore, the role of ROS in apoptosis is paradoxical (Simon et al 2000). Previous reports demonstrated that the podophyllotoxin derivative, podophyllic acid-[4-(2,2,6,6-tetramethyl-1-piperidyloxy)] hydrazone (GP-1) and its congeners podophyllic acid-[4-(2,2,6,6-tetramet-1-piperidyloxyamine)] hydrazone (GP-1-OH) and podophyllic acid-[4-(2,2,6,6-tetramethylchyl-1-piperidyl)] hydrazone (GP-1-H) had antioxidative effects, and their antineoplastic activity is associated with the antioxidative effects (Li et al 2002; Tian et al 2002). Consistent with this notion, our experiment shows that ODE decreases intracellular ROS, which may account for its antineoplastic activity and apoptosis induction.

Conclusions

In summary, it is presumed that, as with most antineoplastic drugs, ODE possesses antineoplastic effects not by inhibiting proliferation, but by promoting apoptosis. Increasing intracellular $Ca^{2+}$and Mg^{2+}, reducing mitochondrial membrane potential, generating ROS and exchanging cytosolic pH value may affect one another, and then induce apoptosis or anti-apoptosis. It is concluded that the anti-osteosarcoma mechanisms of ODE are attributed to apoptosis through increasing intracellular Ca^{2+} and Mg^{2+} concentrations, and reducing pH value, MMP and ROS.

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