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Abstract: Curcumin (diferuloylmethane), one of the main components of the Indian spice turmeric, is known to possess potent anti-inflammatory and anti-oxidant properties. In addition, curcumin has also been shown to have in vitro and in vivo efficacy against a variety of malignancies. In the current study we examined the cytotoxic effect of curcumin on seven osteosarcoma (OS) cell lines with varying degrees of in vivo metastatic potential. Curcumin inhibited the growth of all OS cell lines tested with half-maximal inhibitory concentration values ranging from 14.4 to 24.6 microM. Growth inhibition was associated with a dose dependent increase in the number of apoptotic cells and accumulation of cells in the G(2)/M phase of the cell cycle. Curcumin treatment also resulted in cleavage of caspase-3 and poly adenosine diphosphate-ribose polymerase. Moreover, curcumin treatment was associated with an increase in cellular levels of the apoptotic B-cell leukemia/lymphoma 2 (Bcl-2)-associated X protein and a decrease in cellular content of the anti-apoptotic protein Bcl-2. In addition, curcumin treatment also inhibited the migration of OS cell lines. These data indicate that the potent cytotoxic activity of curcumin on OS cell lines is mediated by induction of apoptotic processes. Thus, curcumin has potential to be a novel OS chemotherapeutic agent.

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Cytotoxic effects of curcumin on osteosarcoma cell lines

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Summary Curcumin (diferuloylmethane), one of the main components of the Indian spice turmeric, is known to possess potent anti-inflammatory and anti-oxidant properties. In addition, curcumin has also been shown to have *in vitro* and *in vivo* efficacy against a variety of malignancies. In the current study we examined the cytotoxic effect of curcumin on seven osteosarcoma (OS) cell lines with varying degrees of *in vivo* metastatic potential. Curcumin inhibited the growth of all OS cell lines tested with half-maximal inhibitory concentration values ranging from 14.4 to 24.6 μM. Growth inhibition was associated with a dose dependent increase in the number of apoptotic cells and accumulation of cells in the G_2/M phase of the cell cycle. Curcumin treatment also resulted in cleavage of caspase-3 and poly adenosine diphosphate-ribose polymerase. Moreover, curcumin treatment was associated with an increase in cellular levels of the apoptotic B-cell leukemia/lymphoma 2 (Bcl-2)-associated X protein and a decrease in cellular content of the anti-apoptotic protein Bcl-2. In addition, curcumin treatment also inhibited the migration of OS cell lines. These data indicate that the potent cytotoxic activity of curcumin on OS cell lines is mediated by induction of apoptotic processes. Thus, curcumin has potential to be a novel OS chemotherapeutic agent.

Keywords Osteosarcoma · Curcumin · Apoptosis

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Introduction

Osteosarcoma (OS) is a primary malignant bone tumor that typically affects children and young adults and is an extremely aggressive disease that is associated with a high degree of lung metastases [1]. Consequently, patients have historically been faced with a poor prognosis. In fact, 15–20% of patients present with radiographically detectable metastatic lesions at the time of diagnosis and 80% of patients with localized tumors have microscopic foci [2]. Prior to the introduction of systemic chemotherapy, osteosarcoma was treated with either amputation and/or radiation therapy. However, the overall 2-year survival rates after such treatment regimens ranged between 15–20% [3, 4]. Fortunately, several different clinical studies demonstrated a significant improvement in patient outcome when chemotherapy was administered before and after surgical resection [5, 6]. Using this regimen 5-year survival rates are now ~70% in patients who present with non-metastatic disease or clinically undetectable metastatic lesions at diagnosis. By contrast, the addition of chemotherapy to surgical resection has not been able to improve the outcome of patients who present with metastatic disease at diagnosis and thus, the 5-year survival rate for these patients remains at only 20% [1].

The most effective chemotherapeutic agents for OS treatment include high-dose methotrexate, cisplatin and doxorubicin [1]. The introduction of additional chemotherapeutic agents, which have been shown to have anti-neoplastic activity against osteosarcoma, such as ifosfamide or etoposide, have unfortunately not been able to significantly improve upon the current 70% 5-year survival rate for patients with non-metastatic disease [1]. Moreover, patients who relapse following administration of currently approved agents have few other chemotherapeutic options

and have significantly lowered survival. Thus, it appears that chemotherapeutic efficacy and patient survival has reached a plateau with currently available chemotherapeutic agents. The use of high-dose methotrexate, cisplatin, doxorubicin and/or etoposide and ifosfamide is also associated with both acute and long-term toxicities [7–9]. Therefore, the development and/or discovery of novel chemotherapeutic agents that improve survival rates and decrease toxicity are of the utmost importance.

Curcumin (diferuloylmethane), a polyphenol, is one of the main components of the Indian curry spice turmeric [10, 11] and is known to be a powerful anti-oxidant with strong anti-inflammatory properties [12, 13]. Recently, curcumin has also been shown to possess potent anti-neoplastic activity (reviewed in [14, 15]) against a number of tumors including prostate, breast and colon cancer [16–18]. To date the exact mechanism(s) underlying curcumin's anti-neoplastic activity has yet to be determined. However, curcumin has been shown to affect numerous signaling pathways and the curcumin-induced cytotoxicity has been hypothesized to vary among cell types [14]. Yet, curcumin has demonstrated *in vivo* efficacy in a number of tumor models and it has recently been successfully tested in Phase I clinical trials for the treatment of colon cancer [19]. Consequently, we were interested in evaluating the anti-cancer effects of curcumin on human osteosarcoma cell lines *in vitro*.

Materials and methods

Cell lines and reagents

The human OS cell lines SAOS-2 (HTB-85), U2OS (HTB-96), HOS and 143B cells were obtained from the American Type Culture Collection (Rockville, MD, USA). LM5 cells were kindly provided by E.S. Kleinerman (M.D. Anderson Cancer Center, Houston, TX, USA). Hu09 and Hu09 m132 cells were provided by Dr. M. Tani (National Cancer Center Hospital, Tokyo, Japan), MG-63 cells were provided by Dr. G. Sarkar (Mayo Clinic, Rochester, MN, USA) and MG-63 M8 cells were provided by Dr. W.T. Zhu (Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China). Normal fibroblasts were obtained from Coriell Cell Repositories (Camden, NJ, USA). All cell lines, except Hu09 and Hu09 m132 were cultured in Dulbecco's Modified Eagle Medium (4.5 g/l glucose)/Ham F12 (1:1; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 1 unit/ml penicillin G, and 1 μg/ml streptomycin. Hu09 and Hu09 m132 cells were cultured in Roswell Park Memorial Institute medium supplemented with 10% FCS, 1 unit/ml penicillin G, and 1 μg/ml streptomycin. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Z-VAD-FMK, a pancaspase inhibitor, was purchased from Becton, Dickinson and Co. (BD) Pharmingen (San Diego, CA, USA). Curcumin was purchased from Sigma-Aldrich Chemical Co. (Buchs, Switzerland).

Cytotoxicity assay

Three thousand cells per well were plated in 96-well plates and allowed to adhere overnight. Various concentrations of curcumin were added the following day. Cytotoxicity was measured 72 h later with water-soluble tetrazolium salt (WST-1) reagent (Roche, Mannheim, Germany), as previously described [20]. Percentage growth inhibition was calculated by dividing the absorbance of curcumin treated cells by that of untreated (control) cells and multiplying by 100. Statistical differences were determined with the Student's *t*-test.

Cell viability assay

Cell viability was measured using Guava Viacount reagent and analyzing the cells on a Guava EasyCyte machine (Guava Technologies Inc., Hayward, CA, USA). Cells (1.5×10^5) were plated in six-well plates and allowed to adhere overnight. The following day curcumin was added at indicated concentrations to the medium. Twenty-four hours later the cell culture medium was removed, cells were washed 1× with phosphate-buffered saline (PBS) and trypsinized. The cells were then washed 1× with PBS and resuspended in 500 μl of PBS. A 20 μl aliquot of cell suspension was then incubated with 180 μl of Viacount reagent for 5 min at room temperature (RT). Cells were then analyzed on a Guava EasyCyte machine using the Viacount Acquisition Module (Guava Technologies Inc., Hayward, CA, USA).

Cell cycle analysis

One hundred fifty thousand cells were plated in six-well plates and allowed to adhere overnight. Indicated curcumin concentrations were added the following day. Following a 24 h incubation, the cell culture medium was removed, cells were washed 1× with PBS and then trypsinized. After trypsinization cells were washed 1× with cold PBS. Cells were then resuspended in 300 μl of cold PBS and 1 ml of ice cold ethanol was added dropwise while vortexing. Cells were then incubated overnight at −20°C. The following day cells were washed 1× with cold PBS. Cells were then resuspended in 500 μl of fluorescence-activated cell sorting solution (38 mM NaCitrate pH 7.5, 69 μM propidium iodide (PI), 10 μg RNase) and incubated in the dark at 37°C for 30 min. Samples were then analyzed on a Guava

EasyCyte machine using the Cell Cycle Acquisition Module (Guava Technologies Inc., Hayward, CA, USA).

Detection of apoptosis

Microscopic analysis

Apoptosis cells were stained with an annexin-V-fluorescein isothiocyanate (FITC) detection kit and necrotic cells were visualized by PI staining (BD Pharmingen, San Diego, CA, USA). One hundred fifty thousand cells were plated in six-well plates and allowed to adhere overnight. Indicated curcumin concentrations were added the following day. Twenty-four hours later the cell culture medium was removed and the cells were washed 1× with PBS and then 1× with annexin binding buffer. The cells were then incubated for 15 min at RT with annexin-V-FITC and PI in annexin binding buffer. The cells were washed 1× and visualized using an Eclipse E600 microscope (Nikon, Egg, Switzerland) equipped with a ×10 objective, a Nikon Y-FL fluorescence illumination with appropriate filters and a Kappa DX20 camera (Kappa opto-electronics GmbH, Gleichen, Germany).

Guava EasyCyte analysis

Apoptosis was also examined with a Guava Nexin Kit containing annexin-V-PE and 7-amino-actinomycin D (7-AAD) for staining of apoptotic and necrotic cells, respectively (Guava Technologies Inc., Hayward, CA, USA). Briefly, 150,000 cells were plated in six-well plates and allowed to adhere overnight. Curcumin was added the following day at indicated concentrations. Twenty-four hours later the cell culture medium was removed and the cells were washed 1× with PBS and trypsinized. The cells were then washed 1× with annexin binding buffer and then incubated for 20 min on ice in 50 μl annexin binding buffer containing annexin-V-PE and 7-AAD. Following incubation, 450 μl of Nexin buffer was added to each tube and the cells were then analyzed on a Guava EasyCyte machine using the Nexin Acquisition Module (Guava Technologies Inc., Hayward, CA, USA).

Wound healing migration assay

Cells were seeded in 24-well plates at approximately 40% of confluence. At confluency, a wound (0.6–1 mm wide and approximately 1 cm in length) was applied with a sterile pin. Cell debris was removed by washing twice with cell culture medium. A homogenous wound area free of cell debris was then marked under the microscope (Nikon Diaphot) with a circle using the Nikon object marker device. The widths of the wounds were measured immediately after wounding (D_o) in the middle of the circle using a Nikon ocular with a graded 1 mm scale. After incubation at 37°C for 24 h in the absence and presence of indicated concentrations of curcumin, the wound widths were measured again (D_t) in the marked area and the migratory rate (μm/h) was calculated by the equation $D_o - D_t/2 \times 24$ h.

Immunoblotting

Cells (1×10^6) were lysed on ice with lysis buffer consisting of 50 mM trishydroxymethylaminomethane pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS). Lysates were cleared of insoluble material by centrifugation at 14,000 rpm, 4°C for 10 min. Protein content was measured with a standard Bradford assay and 80 μg of total protein was resolved by SDS-polyacrylamide gel electrophoresis. Resolved proteins were then transferred to Immobilon-P membranes for immunoblotting. Poly adenosine diphosphate -ribose polymerase (PARP), B-cell leukemia/lymphoma 2 (Bcl-2), Bcl-2-associated X protein (BAX), and caspase-3 were detected using PARP, Bcl-2, BAX and caspase-3 antibodies (Cell Signaling Technology, Beverly, MA, USA). Actin was detected using an actin antibody (Chemicon, Dietikon, Switzerland). Horseradish peroxidase secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

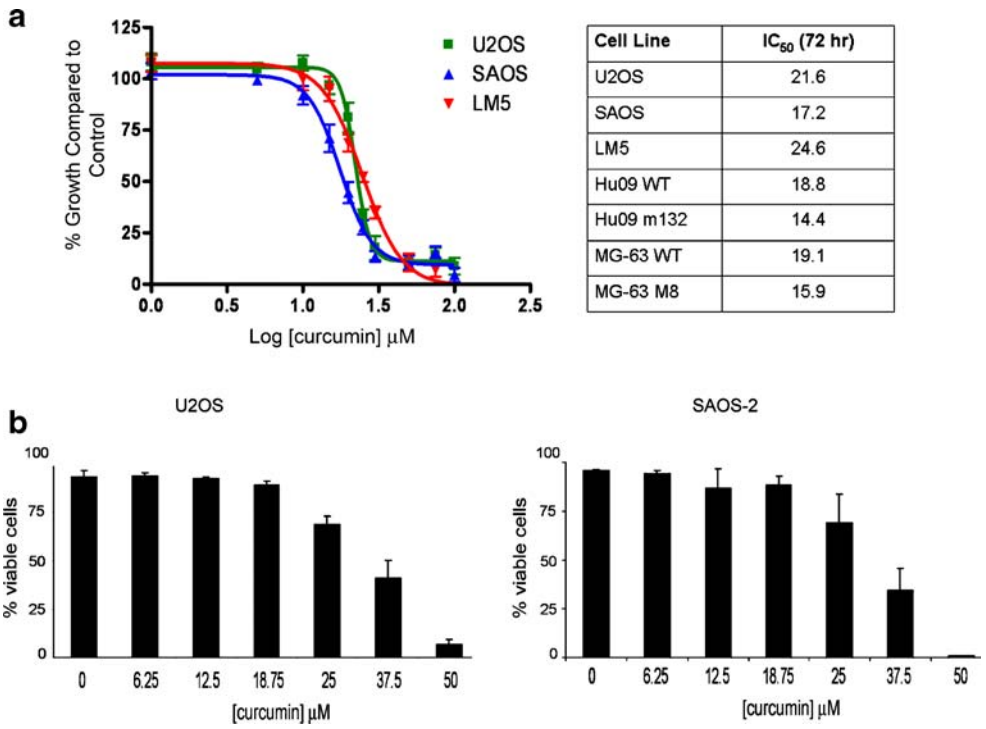
Results

Curcumin inhibits the growth of osteosarcoma cell lines

Incubation with curcumin resulted in growth inhibition of all seven OS cell lines tested and the half-maximal inhibitory concentration (IC_{50}) of curcumin ranged from 14.4 to 24.6 μM (Fig. 1a). The cell lines (Hu09 m132, LM5, MG-63 M8, and 143B) possessing greater *in vivo* metastatic potential than their respective parental cell lines (Hu09 WT, SAOS-2, MG-63 WT and HOS) were found to be relatively equal in their sensitivity to curcumin. Thus, the effect of curcumin appeared to be comparable among all OS cell lines tested. The mechanism(s) of action of curcumin were further assessed, as discussed below, using all cell lines and the results were found to be representative for all cell lines investigated. However, for simplicity, the figures display the results for the U2OS and SAOS-2 cell lines only.

Curcumin treatment decreases OS cell viability

The effect of a drug can be cytotoxic or cytostatic. Thus, we next wanted to determine how curcumin was exerting its growth inhibitory effect on OS cell lines. In initial experiments we examined the effect of curcumin on OS cell line



Cell Line	IC_{50} (72 hr)
U2OS	21.6
SAOS	17.2
LM5	24.6
Hu09 WT	18.8
Hu09 m132	14.4
MG-63 WT	19.1
MG-63 M8	15.9

Fig. 1 Curcumin inhibits the growth of OS cell lines and reduces cell viability. **a** Dose-dependent growth inhibition in indicated OS cell lines following 72-h curcumin treatment. Values represent mean±SEM (n=3). Curcumin IC_{50} values for all cell lines tested are displayed in the accompanying table. **b** U2OS and SAOS-2 cells were treated with increasing concentrations of curcumin. Twenty-four hours later cell viability was analyzed using Guava Viacount reagent. Values represent mean±SD (n=3)

viability using an assay that solely distinguishes viable cells from necrotic cells. As shown in Fig. 1b, treatment of U2OS and SAOS-2 cells with increasing concentrations of curcumin for 24 h resulted in a dose-dependent decrease in viable cells and thus, indicated a cytotoxic effect of curcumin on OS cells.

Curcumin induces apoptosis of OS cell lines

As discussed above, treatment of OS cell lines with curcumin resulted in a decrease in the total number of viable cells. However, the viability assay can only distinguish between viable and necrotic cells and can not distinguish viable cells from early apoptotic cells. Consequently, some early apoptotic cells may be identified as viable cells. Thus, we next wanted to use a more specific apoptosis assay in order to determine whether curcumin was indeed inducing apoptosis of OS cell lines. Early apoptosis is associated with translocation of phosphatidylserine (PS) to the cell surface and can be detected via annexin-V staining due to the high affinity of annexin-V for PS [21]. To assess curcumin-induced apoptosis of OS cell lines, we treated OS cell lines with increasing concentrations of curcumin for 24 h, then incubated the cells with annexin-V-FIT-C and PI and observed the cells microscopically. With all cell lines, a dose-dependent increase in the number of annexin-V-FITC stained apoptotic cells was observed following treatment with increasing concentrations of curcumin, whereas the number of PI-stained necrotic cells remained unchanged (U2OS results shown in Fig. 2a). Curcumin-induced apoptosis was also quantified by flow cytometry using a Guava EasyCyte machine (Fig. 2b–d). Increasing concentrations of curcumin decreased the number of viable cells (annexin-V negative, 7-AAD negative) and increased the number of early apoptotic cells (annexin-V positive, 7-AAD negative cells). When the results of the apoptosis assay are compared to the viability assay, we observed apoptotic cells at concentrations that showed no effect in the viability assay. Thus, some apoptotic cells were indeed identified as viable cells using the viability assay and a more specific apoptosis assay reveals the apoptosis-inducing mechanism of curcumin. An increase in late apoptotic/necrotic cells (annexin-V positive, 7-AAD positive) cells was only observed after treatment with the two highest concentrations of curcumin. The absence of a significant population of late apoptotic/necrotic cells upon microscopic analysis may be attributed to cell detachment.

Curcumin induces cell cycle arrest in the G_2/M phase

Cell cycle distribution of U2OS and SAOS-2 cells, following a 24 h treatment with increasing concentrations of curcumin, was assessed by flow cytometry. Treatment with curcumin resulted in a dose-dependent increase in the number of cells in the G_2/M phase and a decrease in the number of cells in the G_1 phase indicating a curcumin-induced G_2/M phase arrest (Fig. 3). Although a G_2/M phase arrest does not always correlate with an induction of apoptosis, in this case the G_2/M phase arrest results

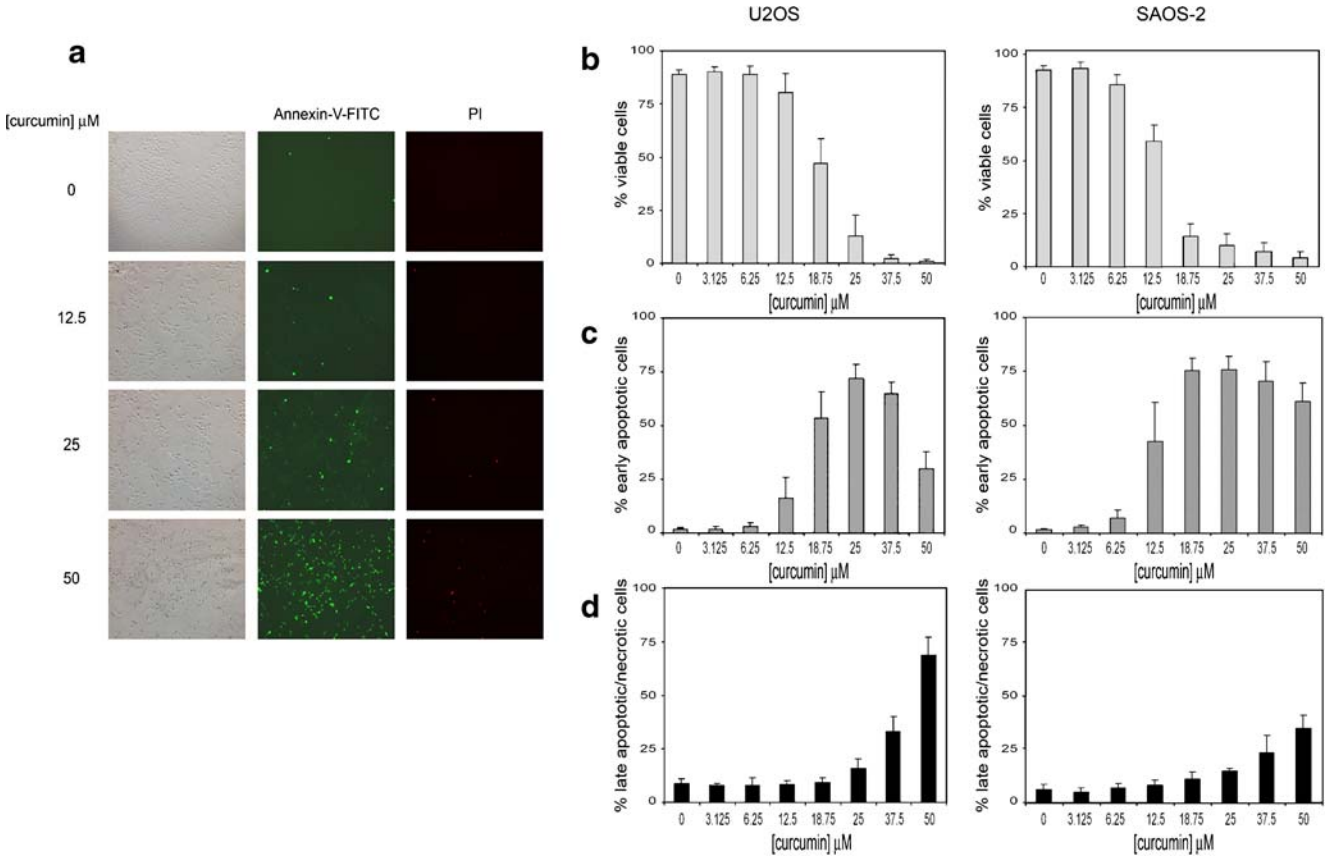


Fig. 2 Curcumin induces apoptosis of OS cells. **a** U2OS cells were treated with increasing concentrations of curcumin. Twenty-four hours later cells were stained with annexin-V-FITC and PI to visualize apoptotic and necrotic cells, respectively. Figures are representative of three independent experiments. **b**–**d** U2OS and SAOS-2 cells were treated for 24 h with indicated curcumin concentrations and 24 h later cells were stained with Annexin-V-PE and 7-AAD and analyzed by flow cytometry. **b** displays the number of viable cells (Annexin-V negative, 7-AAD negative cells), **c** displays the number of early apoptotic cells (Annexin-V positive, 7-AAD negative) and **d** displays the number of late apoptotic/necrotic cells (Annexin-V positive, 7-AAD positive cells). Values represent mean±SD (n=3)

correlate with the observed curcumin-induced apoptosis in Fig. 2c.

Effect of curcumin on apoptotic signaling pathways

Caspases, a family of cysteine acid proteases, are proteolytically activated and then cleave and activate other caspases or cleave and thereby inactivate key cellular proteins such as PARP [22]. Treatment of U2OS and SAOS-2 cells with indicated concentrations of curcumin resulted in a dose-dependent decrease in procaspase-3 and a dose-dependent increase in PARP cleavage (Fig. 4a). A 2-h pre-incubation of the cells with the pancaspase inhibitor Z-VAD-FMK prior to curcumin treatment significantly inhibited the decrease in procaspase-3 and PARP cleavage (Fig. 4b).

In addition to activation of the caspase cascade, curcumin has also been shown to decrease cellular levels of the anti-

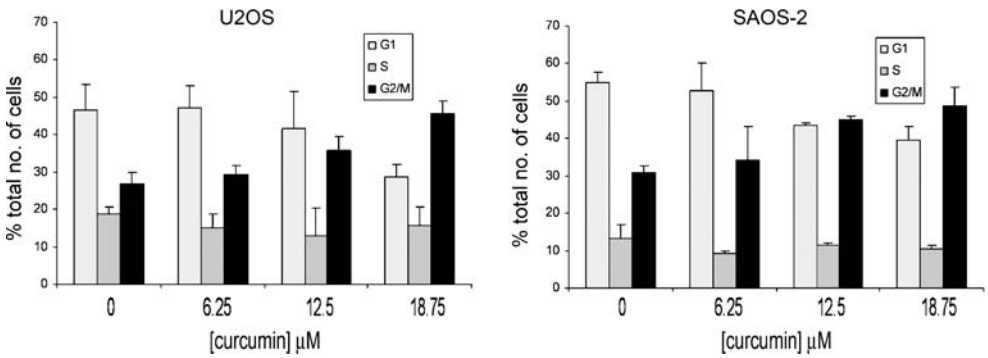


Fig. 3 Curcumin induces G_2/M cell cycle arrest of OS cell lines. U2OS and SAOS-2 cells were treated with increasing concentrations of curcumin. Twenty-four hours later cells were stained with PI and analyzed for DNA content. Values represent mean±SD (n=3)

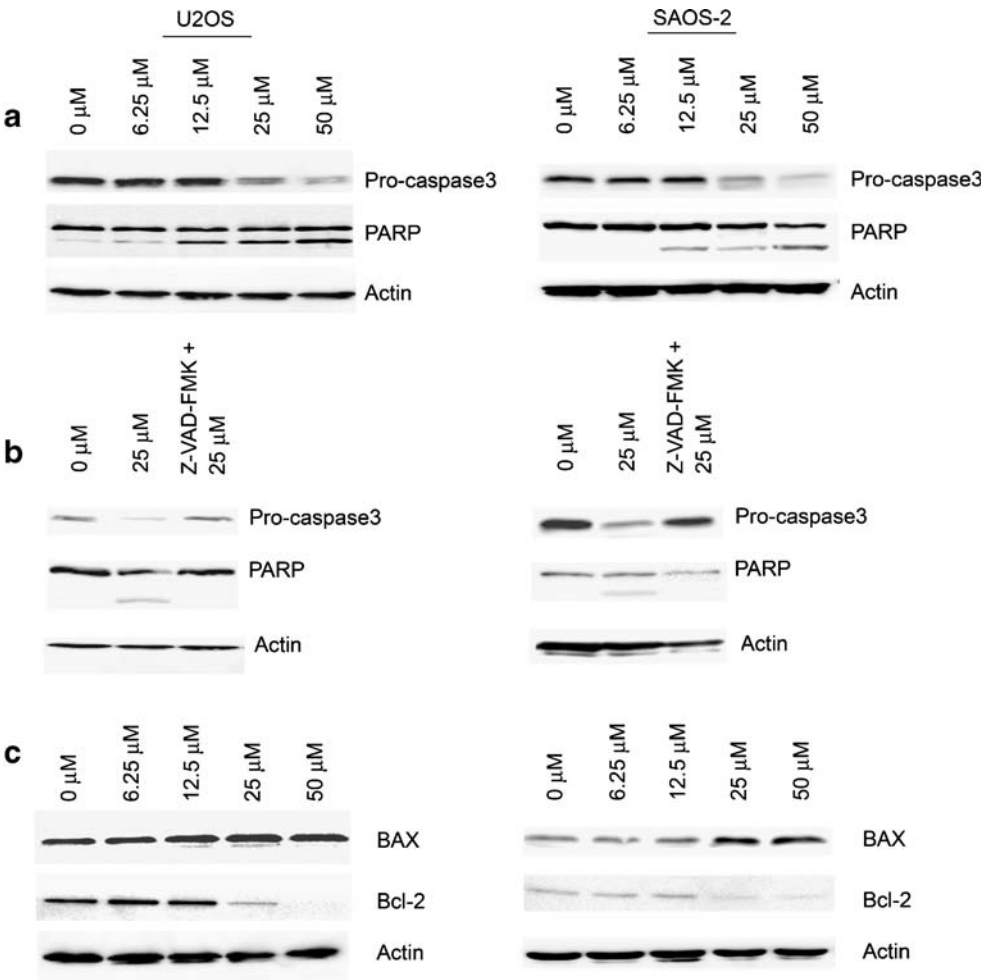


Fig. 4 Effect of curcumin on apoptotic signaling pathways. **a** U2OS and SAOS-2 cells were treated for 24 h with indicated curcumin concentrations and cell lysates were then subjected to immunoblot analysis for procaspase-3, PARP and actin. **b** U2OS and SAOS-2 cells were pre-treated for 2 h with 50 μM of the general caspase inhibitor Z-VAD-FMK prior to curcumin addition. Twenty-four hours following curcumin treatment cells were harvested and cell lysates subjected to immunoblot analysis for procaspase-3, PARP and actin. **c** U2OS and SAOS-2 cells were treated for 24 h with curcumin at indicated concentrations and cell lysates were then subjected to immunoblot analysis for BAX, Bcl-2 and actin

apoptotic protein Bcl-2 and to increase cellular levels of the apoptotic protein BAX [23]. U2OS cells were therefore treated with increasing concentrations of curcumin and the cell lysates were subjected to western blot analysis. As shown in Fig. 4c, curcumin treatment indeed decreased cellular levels of Bcl-2 and increased cellular levels of BAX.

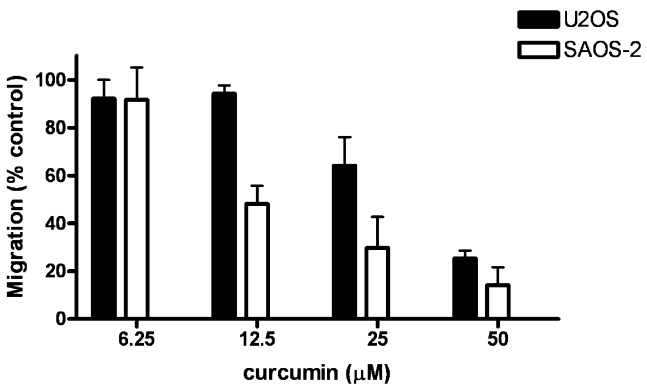


Fig. 5 Curcumin inhibits OS cell line migration. Confluent cells were wounded as described in the "Materials and methods" and incubated at 37°C in the absence and presence of indicated concentrations of curcumin. Wound closure was measured after 24 h and migratory rates calculated as described. Migratory rates of untreated controls were set to 100%. Values represent mean±SEM (n=4)

Curcumin inhibits OS cell line migration

The effect of curcumin on migration and adhesion is well known [24, 25]. Thus, we next wanted to determine if curcumin would inhibit the migration of OS cell lines in the wound healing migration assay. As shown in Fig. 5, wound healing migration of U2OS and SAOS-2 cells was not affected by 6.25 μM curcumin during a 24 h treatment. However, migration of SAOS-2 cells was half-maximally inhibited with 12.5 μM curcumin, while migration of U2OS cells was half-maximally inhibited with 25 μM curcumin. Similar results were obtained with all other cell lines tested. At 50 μM migration was almost completely blocked in all cell lines. At this concentration, but not below all cell lines started to partly detach and had a rounded appearance.

Curcumin can be used in combination with cisplatin

In order to determine whether curcumin could be used in combination with current OS chemotherapeutic agents, U2OS cells were incubated with increasing concentrations of doxorubicin or cisplatin in the absence and presence of

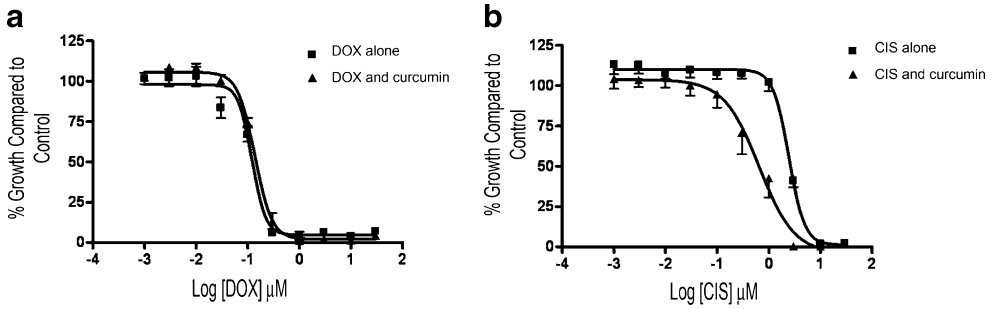


Fig. 6 Curcumin demonstrates an additive effect with cisplatin. **a** U2OS cells were treated for 72 h with either increasing concentrations of doxorubicin alone (*filled square*) or in combination with 15 μM curcumin (*filled triangle*). Viable cells were then determined with a WST-1 assay. Values represent mean±SEM (n=3). **b** U2OS cells were treated with either increasing concentrations of cisplatin alone (*filled square*) or in combination with 15 μM curcumin (*filled triangle*). Viable cells were then determined with a WST-1 assay. Values represent mean±SEM (n=3)

15 μM curcumin, a concentration produces a 10% growth inhibition after a 72 h incubation. The combined treatment of cells with curcumin and doxorubicin did not have an additive effect compared to treatment with either drug alone (Fig. 6a). However, additive cytotoxicity was observed with curcumin and cisplatin (Fig. 6b).

Discussion

The discovery and development of novel chemotherapeutic agents that can improve OS survival rates and/or lower the occurrence of toxic side effects experienced with currently approved agents is of the utmost importance. Recently, several reports have described the potent anti-neoplastic activity of curcumin against several diverse types of malignancy. Consequently, significant interest in curcumin as a potential anti-cancer drug has been generated and curcumin has recently been extensively tested in Phase I clinical trials [26]. In this study we examined the anti-neoplastic activity of curcumin against OS cell lines. Initial experiments demonstrated that all OS cell lines tested were remarkably sensitive to the effects of curcumin with IC_{50}s ranging from 14.4–24.6 μM. Similar IC_{50} values were reported following curcumin treatment of HeLa and MCF-7 cell lines [27] and thus, our inhibitory concentrations are within previously reported values.

The anti-proliferative activity of curcumin has previously been attributed to curcumin-induced apoptosis [28–30]. Our step-by-step examination using viability and apoptosis assays revealed that curcumin indeed induces apoptosis of OS cell lines. Investigation of the signaling pathways activated by curcumin confirmed that curcumin induces apoptosis of OS cell lines and that caspases are involved in this process. However, curcumin has been previously shown to affect numerous diverse signaling pathways (reviewed in [14]). Moreover, the apoptotic process is known to involve an orchestrated series of signaling events and thus, curcumin-induced apoptosis of OS cell lines may involve additional signaling pathways. Two proteins that have previously been shown to be involved in curcumin-induced apoptosis are Bcl-2 and BAX. Proteins of the Bcl-2 family are key regulators of apoptosis while BAX belongs to the pro-apoptotic group [31]. Bcl-2 exerts its anti-apoptotic effects via inhibition of mitochondrial cytochrome c release. By contrast, BAX asserts a pro-apoptotic effect by interacting with membrane pore proteins to increase cytochrome c release [32, 33]. Curcumin treatment of diverse cell types has recently been shown to result in decreased Bcl-2 cellular levels and increased cellular levels of BAX [34, 35]. Moreover, Anto et al. [36] and Shankar and Srivastava [23] demonstrated that curcumin-induced regulation of Bcl-2 and BAX is essential for curcumin-induced apoptosis. In the current study, curcumin treatment of OS cell lines also resulted in decreased and increased cellular levels of Bcl-2 and BAX, respectively. Consequently, these findings indicate that Bcl-2 and BAX are also critical regulators of curcumin-induced apoptosis in OS cell lines.

The ability of curcumin to inhibit cell migration and adhesion is well known [24, 25]. Indeed, in the current study curcumin treatment of OS cell lines was also found to inhibit cell migration. The exact mechanism of curcumin-induced cell migration inhibition is not known but curcumin has been shown to affect proteins involved in cell-cell adhesion such as ß-catenin and E-cadherin [14]. Based on curcumin's ability to inhibit cell migration and adhesion *in vitro*, it is also believed that curcumin may be able to inhibit the migration of established malignancies *in vivo* and thus, decrease the formation of metastases.

According to current European and American Osteosarcoma Study Group treatment guidelines, OS patients typically receive 10 weeks of neo-adjuvant therapy, undergo surgical resection with wide margins and then receive adjuvant therapy based on their histological response to neo-adjuvant treatment. In order to improve overall patient survival, novel agents must either be sufficiently potent to take the place of existing therapies

or must be compatible with previously established regimens. In this study we examined the compatibility of curcumin in combination with doxorubicin and cisplatin and of note, the effect of curcumin was shown to be additive with the effect of cisplatin. By contrast, the effect of curcumin was not found to be additive with the effect of doxorubicin, yet curcumin did not inhibit the cytotoxic effect of doxorubicin. The combination effect of curcumin with cisplatin and lack of an additive effect with doxorubicin was also reported by Notarbartolo et al. [37] with human hepatic cancer cells. In this report, the authors discuss that curcumin is known to interfere with nuclear factor-kappa B (NF-κB) activation and that other reports indicate that doxorubicin requires NF-κB activation in order to induce its cytotoxic effect. Consequently, it has been suggested that curcumin may diminish the antitumor effect of doxorubicin. Nonetheless, *in vitro* curcumin appears to be compatible with agents used for treatment of osteosarcoma and supports further investigation into its potential for clinical application. However, pre-clinical and clinical investigations must pay particular attention to synergistic and sub-additive effects of curcumin when used in combination with current therapeutic agents.

The current study and numerous additional studies have demonstrated the potent anti-neoplastic properties of curcumin. Furthermore, animal models and pre-clinical trials have reported no adverse effects following administration of curcumin. However, widespread clinical application of curcumin has been limited due to poor aqueous solubility and low systemic bioavailability. Nevertheless, enthusiasm for clinical use of curcumin continues and recently Bischt et al. described the development and successful testing of aqueous soluble nanocurcumin *in vitro* [38]. Consequently, optimism continues for the clinical potential of curcumin for treatment of OS and various other malignancies.

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