Evaluation of Quercetin as a Potential Drug in Osteosarcoma Treatment

KERSTEN BERNDT[1*], CARMEN CAMPANILE[1*], ROMAN MUFF[1], EMANUEL STREHLER[2], WALTER BORN[1] and BRUNO FUCHS[1]

[1]*Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, University of Zurich, Zurich, Switzerland;*
[2]*Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, U.S.A.*

Abstract. *Background: Osteosarcoma is the most common malignant bone tumor in children and young adults. Since the introduction of chemotherapy, the 5-year survival rate of patients with non-metastatic osteosarcoma is ~70%. The main problems in osteosarcoma therapy are the occurrence of metastases, severe side-effects and chemoresistance. Antiproliferative and apoptotic effects of quercetin were shown in several types of cancers, including breast cancer and lung carcinoma. Materials and Methods: The present study investigates the cytotoxic potential of quercetin, a dietary flavonoid, in a highly metastasizing human osteosarcoma cell line, 143B. Results: We found that quercetin induces growth inhibition, G_2/M phase arrest, and apoptosis in the 143B osteosarcoma cell line. We also observed impaired adhesion and migratory potential after the addition of quercetin. Conclusion: Since quercetin has already been shown to have low side effects in a clinical phase I trial in advanced cancer patients, this compound may have considerable potential for osteosarcoma treatment.*

Osteosarcoma is a primary malignant bone tumor that typically affects children and young adults. Current treatment includes neoadjuvant multi-agent chemotherapy and surgical resection of the primary tumor (1, 2). However, despite different approaches using conventional chemotherapy to improve the survival of patients with metastatic disease, the 5-year survival rate for these patients remains at 20%. Additionally, the use of common chemotherapeutic agents such as high-dose methotrexate, cisplatin, doxorubicin and/or etoposide and ifosfamide frequently causes both acute and long-term toxicity (2).

Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the most studied dietary flavonoids. Several *in vitro* studies have suggested antioxidant, anti-inflammatory, antiproliferative and apoptotic effects of quercetin on cancer cells (3, 4). In various tumor cell lines, cell growth was significantly inhibited by quercetin treatment. Half-maximal inhibitory concentrations (IC_{50}) ranged from 7 nM in the MNT1 melanoma cell line to over 100 μM in HTB43 head and neck cancer cells. Two recent studies on the osteosarcoma cell lines MG63, U2OS and HOS showed that quercetin is effective in blocking cell proliferation, with an IC_{50} of between 22 and 160 μM (5, 6).

The molecular mechanism of quercetin action in human breast carcinoma cell lines includes down-regulation of mutant *p53* and heat-shock proteins, with consequent G_2/M phase cell-cycle arrest (7, 8). Consistent with this, quercetin was also shown to induce G_2/M phase cell-cycle arrest as well as apoptotic and necrotic cell death in the U138MG human glioma cell line (9). Furthermore, treatment of human leukemia T-cells and gastric cancer cells with quercetin resulted in a late G_1 block associated with reduced DNA replication and a delay in cell division (8, 10).

In the human breast cancer cell line MDA-MB-231, quercetin-dependent cell death was shown to occur *via* activation of mitochondrial apoptotic pathways (11). Comparable results were reported for MG63, HOS and U2-OS osteosarcoma cell lines where a reduction of pro-caspase-3 and an increase in the amount of cleaved poly (ADP-ribose) polymerase (PARP) were observed, suggesting that quercetin-induced apoptosis is mainly caspase-dependent (5, 6, 12).

Unfortunately, none of the above studies used osteosarcoma cell lines suitable for the generation of a

*These Authors contributed equally to this work.

Correspondence to: Bruno Fuchs, MD, Ph.D., Department of Orthopedics, Balgrist University Hospital, Forchstrasse 340, 8008 Zurich, Switzerland. Tel: +41 443861661, Fax: +41 443861669, e-mail: bfuchs@research.balgrist.ch

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mouse model where the effects of quercetin could be tested *in vivo* (13-15). The results were not reproducible when HOS cells were injected intratibially nor when U2-OS were injected subcutaneously. Moreover, none of the above osteosarcoma cell lines, including MG63, exhibited any metastatic properties *in vitro* and have therefore to be considered as being of low or non-metastatic potential. However, osteosarcoma is known as a disease with a high propensity for metastasis to the lung. Consequently, the screening of novel drugs for the treatment of osteosarcoma should be carried out with osteosarcoma cell lines, which, as closely as possible, reproduce the human disease in mice.

Therefore, in the present study, we decided to evaluate the anticancer properties of quercetin in the well-characterized human 143B osteosarcoma cell line, which upon intratibial injection in severe combined immunodeficiency (SCID) mice, reproducibly forms primary bone tumors that predominantly metastasize to the lung (16).

Materials and Methods

Cell line. The human osteosarcoma cell line 143B was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-HamF12 (1:1) medium (PAA GmbH, Coelbe, Germany) supplemented with 10% heat-inactivated fetal calf serum as described by Arlt *et al*. (17).

Cell metabolic assay (WST-1). 143B cells were seeded at a density of 3,000 to 7,800 cells per well in 96-well-plates, depending on the time point after cell seeding at which the metabolic activity was measured. After seeding, the cells were allowed to adhere for at least 10 h. Increasing concentrations of quercetin (Sigma, St Louis, MO, USA) or vehicle dimethyl sulfoxide (DMSO) were added the following day and the cells incubated for 12, 24 and 36 h. The metabolic activity of cells was subsequently measured with the water-soluble tetrazolium salt WST-1 reagent (Roche, Mannheim, Germany), as described previously (18). A parallel 96-well plate was prepared with medium and different concentrations of quercetin in order to subtract the background staining contributed by quercetin-alone. The percentage of metabolically active cells was calculated, after subtraction of quercetin concentration-dependent background staining, by dividing the absorbance of quercetin-treated cells by that of vehicle-treated cells and multiplying by 100.

Cell proliferation and viability assay. The cell number was determined with the Guava Viacount reagent and samples were analyzed on a Guava EasyCyte machine (Guava Technologies Inc., Hayward, CA, USA). Briefly, 100,000 143B cells per well were plated in 6-well plates and allowed to adhere for 6 h. Quercetin or vehicle (DMSO) was then added to the medium at the indicated concentrations. Forty-eight hours later, the cell culture medium was collected, and the cells were washed with phosphate buffered saline (PBS) and trypsinized. The cell pellet was collected and resuspended in 500 μl PBS. A 20-μl aliquot of cell suspension was then incubated with 180 μl of Viacount reagent for 5 min at room temperature (RT). The cell number was determined using the Viacount Acquisition Module on a Guava EasyCyte machine and the cell viability was also assessed in the same sample (Guava Technologies Inc.).

Cell division assay. Doubling time of 143B cells was calculated by plating 50,000 cells per 25-cm^2 dish on day 0 and counting the cells with a hemocytometer at different time points. Doubling was calculated according to the formula $t_{1/2}=\ln 2/\gamma$ where $t_{1/2}$ is the doubling time and γ is the slope of the linear regression curve.

A cell division assay based on the serial dilution of the fluorescence intensity of the stable intracellular fluorochrome carboxyfluorescein succinimidyl ester (CFSE) (Guava Cell Growth Assay, Millipore, Zug, Switzerland) was optimized to study cell division in 143B osteosarcoma cells. For the staining procedure, approximately 5×10^6 cells were diluted in 500 μl PBS containing 0.1% bovine serum albumin (BSA). An aliquot of 140 μl was kept as a negative control for the CFSE staining. The remaining 360 μl were incubated with 25 μM CFSE diluted in anhydrous DMSO at 37˚C for 15 min. The staining control was incubated with 6% anhydrous DMSO and treated equally. Following staining, the cell suspension was diluted in 10 ml medium and incubated for 10 min at RT. The cells were then washed twice with medium, resuspended in medium and the cell number adjusted to 150,000 cells per ml. One milliliter cell suspension per well was plated in a 6-well plate. Cells were allowed to adhere for 6 h and were then treated for 18 h with quercetin concentrations (25 or 50 μM) or vehicle. One sample of stained and non-stained cells was harvested prior to the 18-h incubation and fixed with 100% ice-cold ethanol as positive and negative staining control, respectively.

Cell-cycle assay. Five hundred thousand cells were plated in 25 cm^2 flasks and allowed to adhere for approximately 8 h. Quercetin (10 or 25 μM) or vehicle was then added, and the cells were incubated for another 24 h. Following incubation, the cell culture medium was collected, and the cells were washed once with PBS and then trypsinized. Cell pellets were washed once with 1 ml cold PBS and the cells were resuspended in 300 μl of cold PBS. One milliliter of ice-cold ethanol was then added dropwise while vortexing, and the cells were stored at –20˚C overnight. The following day, cells were washed once with cold PBS, resuspended in 500 μl fluorescence-activated cell sorting (FACS) solution [38 mM sodium citrate, 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 BD FACSCalibur machine using the Watson Pragmatic mathematical model (BD Biosciences, CA, USA). The percentage of dead cells was not included in our analysis.

Immunoblotting of apoptosis-related proteins. 143B cells were treated for 36 h with different concentrations of quercetin and the whole-cell protein lysate was then collected, sonicated, lysed and cleared as described earlier (19). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for immunoblotting. PARP, cleaved caspase-7 and cleaved caspase-3 were detected using appropriate specific antibodies (Cell Signaling Technology, Beverly, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal protein loading standard was detected using GAPDH antibody (Santa Cruz, CA, USA). PARP cleavage was inhibited by pre-incubation of the cells with the pan-caspase inhibitor, Z-VAD-FMK (BD Pharmingen AG, Allschwil, Switzerland) for 1 h before quercetin treatment for 36 h.

Adhesion assay. Sub-confluent (80%) cells were incubated with a medium containing quercetin (10, 50 or 100 μM) or 2% DMSO as vehicle. Twelve hours later the cells were detached using 0.05% PBS/EDTA, counted and seeded in triplicates (100 μl/well) in 96-well plates at a density of 50,000 cells/ml. The cells were allowed to adhere for 15 or 30 min. Non-adherent cells were washed away with PBS while adherent cells were fixed in 10% formalin for 10 min and stained with 0.05% crystal violet at room temperature for 15 min. Images were obtained using an AxioCam Mrm camera mounted on a Zeiss Observer Z1 inverted-microscope (Carl Zeiss Microimaging GmbH, Goettingen, Germany) at ×4 magnification: the total area for each image was 3.6 mm^2. The number of adherent cells was determined using the ImageJ software (http://rsb.info.nih.gov/ij) and the percentage of adherent cells was calculated relative to the total number of seeded cells.

Wound-healing migration assay. Fifty thousand cells per well were cultured in a 24-well plate for 24 h. Incubation with quercetin (10, 25 or 50 μM) or vehicle was initiated approximately 7 h before wounding. When the cells were confluent, a scratch wound (0.3-1 mm wide and approximately 1 cm in length) was applied with a sterile pin. Cell debris was removed by washing twice with warm cell culture medium containing penicillin, streptomycin and amphotericin (PSA) (Life Technologies, Zug, Switzerland). A homogenous wound area free of cell debris was then marked under the microscope (Nikon AG Eclipse E600; Nikon Corporation, Egg, Switzerland) with a circle using the Nikon object marker device. The widths of the wounds were determined immediately after wounding (D_0) in the middle of the circle using a Nikon ocular with a graded millimeter scale. After incubation for 12-15 h in the absence or presence of quercetin (10, 25 or 50 μM) or vehicle, the wound widths were measured again (D_t) in the marked area and the wound-healing migration rate (μm/h) was calculated by the equation D_0-D_t/2t, where t is the incubation time (12-15 h).

Results

Quercetin inhibits cell growth and viability of 143B osteosarcoma cells. Treatment of the highly metastatic 143B cell line with quercetin resulted in a dose-dependent decrease of viable metabolically active cells (WST-1 assay) in comparison to vehicle-treated control (Figure 1A). A 39% (±11) decrease in metabolic activity was observed as soon as 12 h of treatment under high quercetin concentrations. A maximal decrease in metabolic activity was obtained after a 24 h treatment, with an IC_{50} of about 80 μM of quercetin, and it was indistinguishable from that observed after a 36 h treatment. We next investigated whether the quercetin-dependent reduction of viable cells is caused by cytotoxic or cytostatic effects. To this end, the cell number and viability was determined 48 h following quercetin treatment with the propidium iodide-based Guava Cell Viability Assay. As shown in Figure 1B, the lowest dose of 10 μM quercetin significantly reduced ($p<0.0001$) the cell number to 76% (±6.3%) compared to the control. Interestingly, however, the fraction of viable cells was affected only by the three highest quercetin concentrations (Figure 1C). These results indicate that quercetin initially has cytostatic effects and later causes dose-dependent cell death.

Quercetin causes an arrest of 143B cells in the G_2/M transition of the cell cycle. The experiments described above suggest that quercetin inhibits cell growth. To visualize differences in cell division of quercetin-treated *versus* control cells the CFSE assay was performed. CFSE is a fluorescent cell stain that allows for assessment of cell division, because CFSE fluorescence is equally divided between daughter cells during each cell division (20). The mean fluorescence intensity (MFI) of quercetin-treated and non-treated cells after 18 h in culture was compared to the MFI of cells that were fixed directly after staining. The results showed that after culturing the cells for 18 h, which is close to the doubling time of 143B cells (Figure 2A), the MFI in the stained, vehicle-treated cells dropped to approximately half of the initial value (Figure 2B). Quercetin-treated samples exhibited a dose-dependent reduction in the decrease of the MFI, indicating an inhibition of cell division that is almost complete when quercetin is applied at 50 μM (Figure 2B). In order to investigate the mechanism involved in growth inhibition by quercetin, the cell-cycle distribution of 143B cells following 24-h drug treatment was evaluated by flow cytometry. As shown in Figure 3A, quercetin treatment resulted in a dose-dependent increase in the G_2 fraction and a decrease in the G_1 fraction, indicating a G_2/M phase arrest. Treatment with 25 μM quercetin resulted in a decrease of G_1-phase cells from 34±0.7% in untreated controls to about 16±3.2% ($p<0.05$), and a concomitant increase of G_2/M-phase cells to 39±2.4% compared to 20±2.3% ($p<0.05$) in controls. No significant difference in the percentage of cells in the S phase was observed between these two groups (Figure 3A). The marked G_2/M phase arrest of the cells treated with 25 μM quercetin is clearly visible by the increased G_2 peak in the representative histogram from the flow cytometry analysis (Figure 3B).

Quercetin induces apoptosis in human 143B osteosarcoma cells. The G_2/M phase arrest in quercetin-treated 143B cells described above is followed by cell death *via* activation of the apoptotic signaling pathway (Figure 4A). Immunoblotting of proteins in total cell lysates showed dose-dependent caspase-3, caspase-7 and PARP cleavage following 36 h of quercetin treatment. PARP cleavage was already detectable 24 h after the addition of quercetin to the cells (data not shown). However, PARP was still mostly intact 12 h after the addition of quercetin to the cells, which suggests that apoptotic signaling is activated after the cell-cycle has been arrested. The induction of apoptosis by quercetin was almost completely inhibited by pre-treatment of the cells with the pan-caspase inhibitor Z-VAD-FMK (Figure 4B).

Effect of quercetin on 143B cell adhesion to plastic dishes and wound-healing properties. Since cell adhesion and migration play important roles in the pathogenesis of cancer and

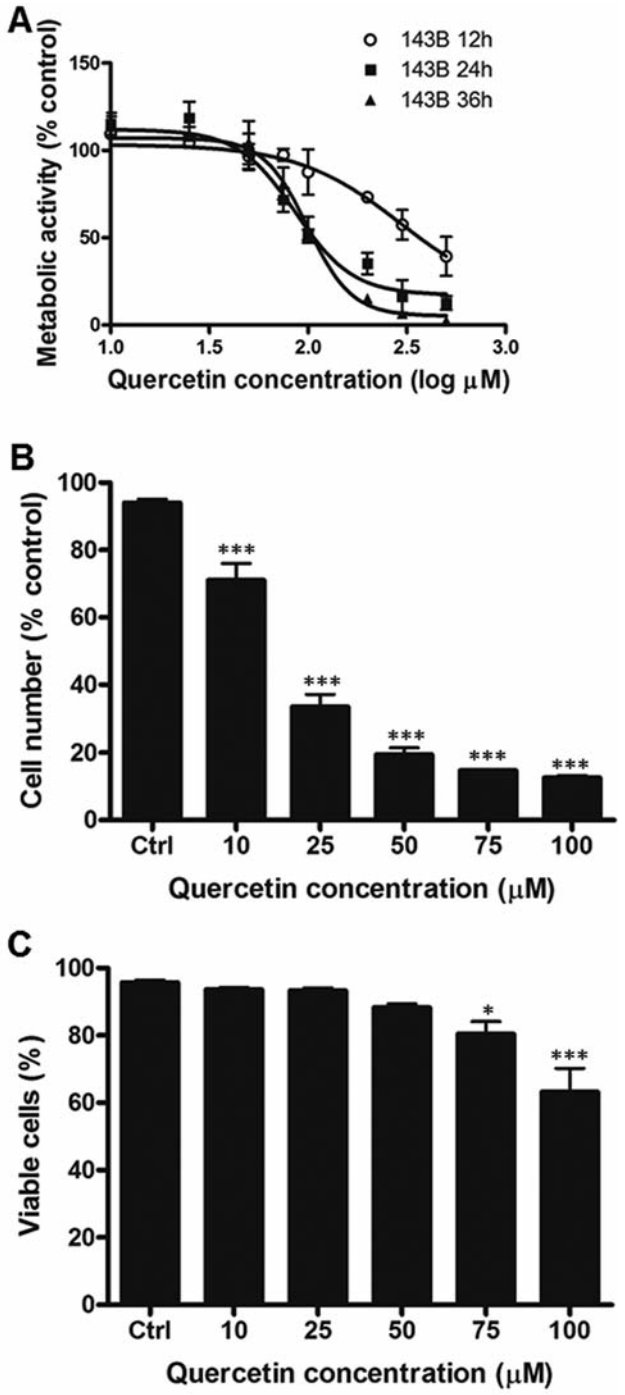

Figure 1. *A: Quercetin inhibits cell metabolism in the 143B osteosarcoma cell line. Cells were treated with the indicated concentrations of quercetin and their metabolic activity-determined after 12, 24 and 36 h as described in Materials and Methods. Values represent the mean±SEM (n=2). B: Effect of quercetin on the number of 143 osteosarcoma cells. Total cell number was determined using Viacount reagent following 48 h quercetin treatment at the indicated concentrations. The total cell number includes the apoptotic, dead and viable cells. Values represent the mean±SEM (n=3). C: Effect of quercetin on cell viability of 143B osteosarcoma cells. Percentage of viable cells is shown in panel (B). Values represent the mean±SEM (n=3).*

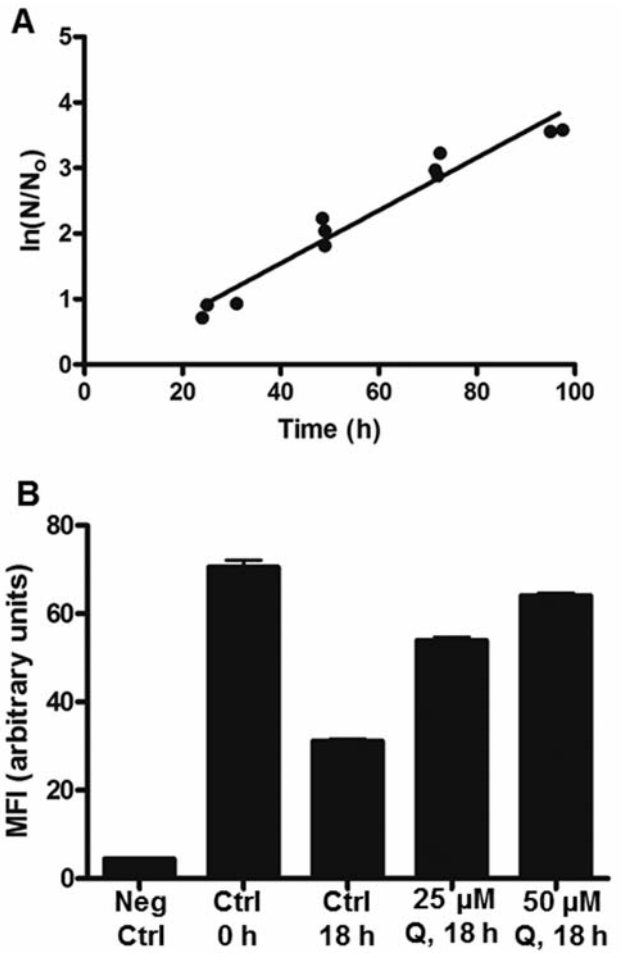

Figure 2. *A: Quercetin treatment inhibits division of 143B cells. Linear regression of the increase in the number of 143B cells over time. The calculated slope is 0.04 h^{-1} and equivalent to a doubling time of 17.2 h. N=number of cells at time t; N_0=number of cells at time 0. B: 143B cells were analyzed with the Guava Cell Growth Assay. References for the median fluorescence intensity (MFI) were freshly-stained (Ctrl 0 h) and non-stained fixed cells (neg Ctrl). The MFI in cells treated with quercetin (25 and 50 μM) for 18 h was higher than in control cells (Ctrl 18 h), suggesting growth inhibition by quercetin in a dose-dependent manner.*

development of metastases, we tested the effect of quercetin on the adhesion and migration of 143B osteosarcoma cells. After exposing 143B cells for 12 h to 50 μM or 100 μM quercetin, the percentage of adherent cells to plastic (evaluated 30 min after plating) decreased significantly to 51±15% ($p<0.05$) and 42±11% ($p<0.01$), respectively, of the number observed with non-treated cells (Figure 5A). When the analysis was performed 15 min after plating, the percentage of adherent cells after treatment with 50 and 100 μM of quercetin was 48±14% and 44±16% of that of non-treated cells, respectively. Statistical analysis was performed on the raw data (cell numbers) and not on the percentage of adherent

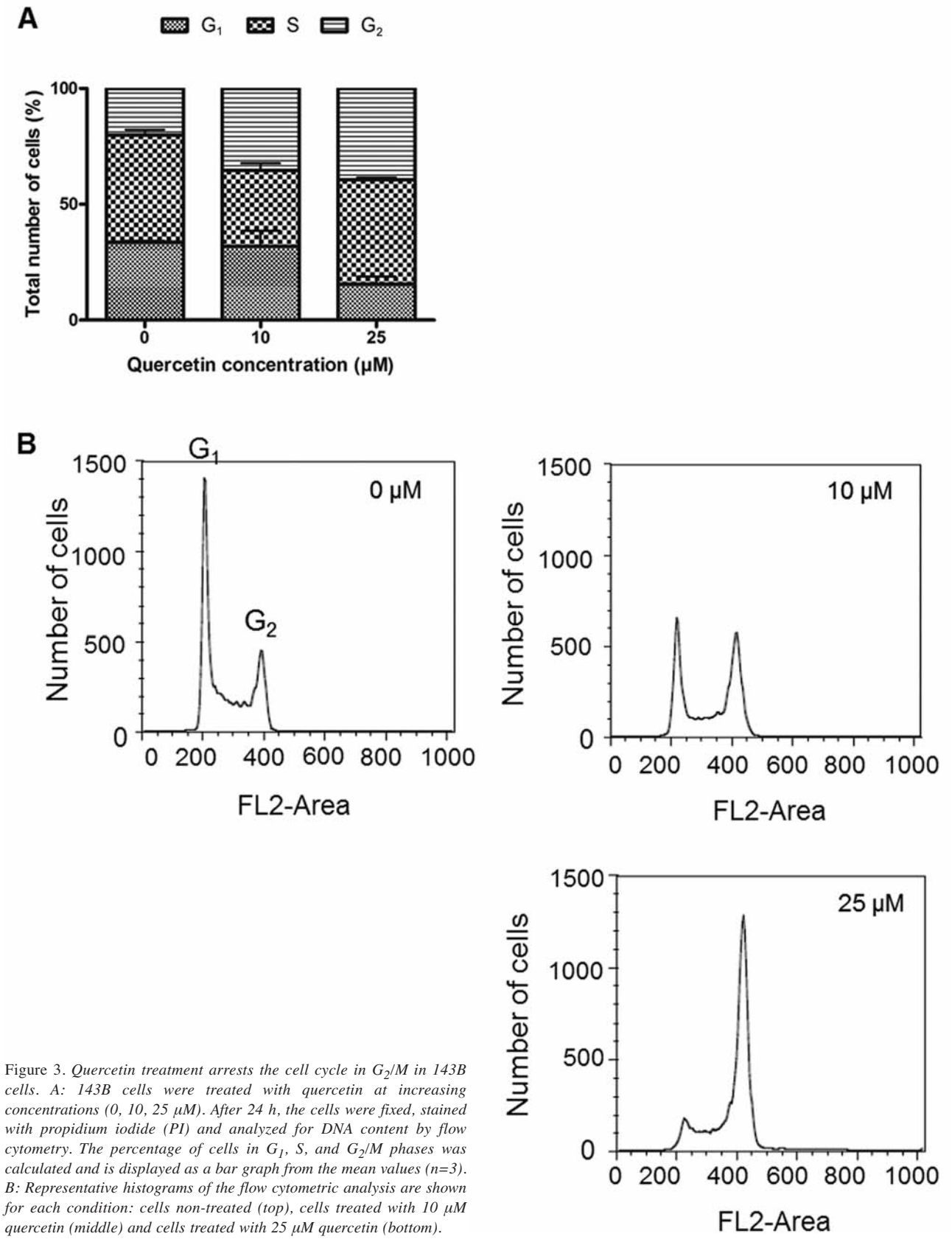

Figure 3. *Quercetin treatment arrests the cell cycle in G_2/M in 143B cells. A: 143B cells were treated with quercetin at increasing concentrations (0, 10, 25 μM). After 24 h, the cells were fixed, stained with propidium iodide (PI) and analyzed for DNA content by flow cytometry. The percentage of cells in G_1, S, and G_2/M phases was calculated and is displayed as a bar graph from the mean values (n=3). B: Representative histograms of the flow cytometric analysis are shown for each condition: cells non-treated (top), cells treated with 10 μM quercetin (middle) and cells treated with 25 μM quercetin (bottom).*

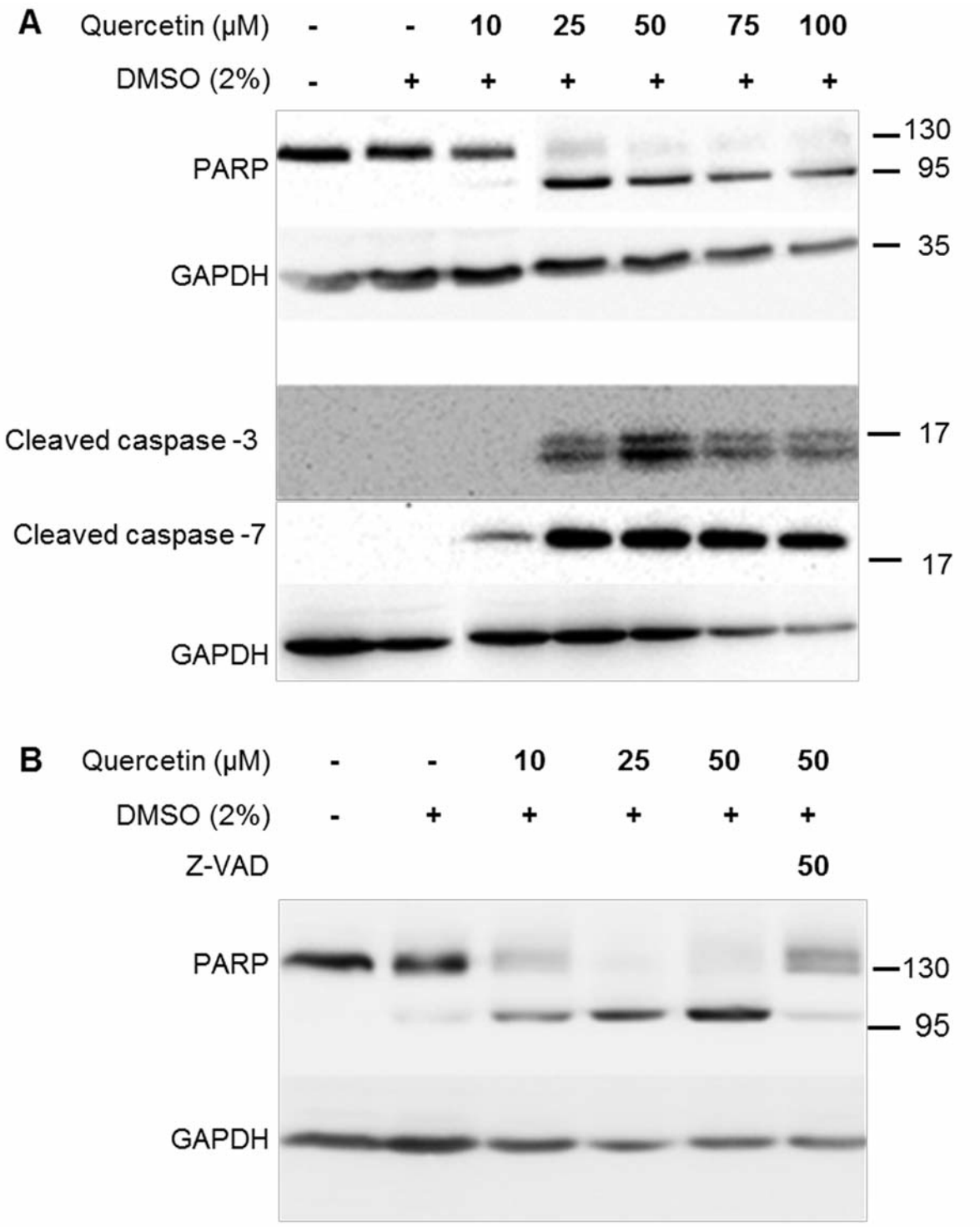

Figure 4. *Biochemical analysis of quercetin-mediated apoptosis in 143B cells. A: Induction of apoptosis following quercetin treatment occurs via caspase-3, -7 and poly (ADP-ribose) polymerase (PARP) cleavage. 143B cells were treated with the indicated quercetin concentrations (0, 10, 25, 50, 75, 100 μM) for 36 h. An aliquot of untreated cells was also kept in 2% dimethyl sulfoxide (DMSO) for 36 h since quercetin incubations were carried out at this concentration of DMSO. Total cell lysates were then subjected to immunoblot analysis for cleaved caspase-3, -7 and PARP. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard for protein loading. Protein molecular masses are indicated in kDa on the right. B: Apoptosis activation is inhibited by the pan-caspase inhibitor Z-VAD. Cells were pre-treated with 50 μM Z-VAD for 1 h prior to quercetin treatment. 143B cells were incubated with quercetin at the indicated concentrations (0, 10, 25, 50 μM) for 36 h. A control incubated with 2% DMSO was also included in this experiment as in panel A. GAPDH was used as an internal standard for protein loading.*

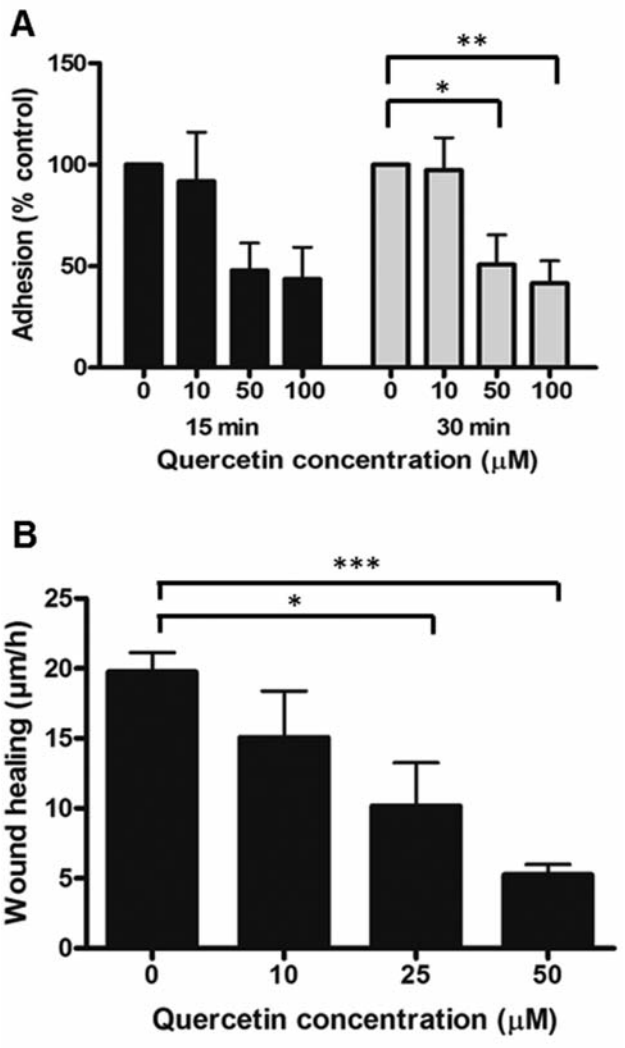

Figure 5. *Quercetin inhibits adhesion and migration of 143B cells in a dose-dependent manner. A: Cells were incubated with quercetin at the indicated concentrations for 12 h. Cells were left adhering in the presence or absence of quercetin for 15 min or for 30 min. The percentage of adherent cells was counted and compared to the percentage of non-treated adherent cells. Values represent the mean±SEM (n=3); *p<0.05; **p<0.01. B: Wound-healing migration assays were performed in the absence or presence of quercetin as indicated. Wound closure was measured after 12 to 15 h of incubation and migration rates were calculated as described in Materials and Methods. Values represent the mean±SEM (n=3); *p<0.05; ***p<0.001.*

cells shown in the bar graphs in Figure 5A. This explains why adhesion 15 min after cell seeding does not yield the same statistical significance *versus* the control as after 30 min, despite the fact that the relative differences in the percentage of adherent cells are virtually the same (Figure 5A). The wound-healing assay showed that quercetin inhibited cell migration in a dose-dependent manner (Figure 5B). The IC_{50} was determined to be approximately 25 μM quercetin. At this concentration, the migration rate was inhibited by two-fold compared to non-treated cells in a statistically significant manner ($p<0.05$). At the highest applied quercetin concentration of 50 μM, the migration rate was reduced by 3.7-fold compared to non-treated controls (Figure 5B; $p<0.0005$).

Discussion

In this study, we evaluated the effect of the flavonoid quercetin *in vitro* on a highly-metastasizing human osteosarcoma cell line, 143B. Since the introduction of neoadjuvant chemotherapy in the late 1970s, no new compound has been found to be equally or more effective for osteosarcoma treatment than methotrexate, doxorubicin and cisplatin (21-23). The effect of quercetin has already been studied in MG63, HOS and U2OS cells (5, 6), however, in comparison to these cell lines, only 143B cells are able to generate a reliable and reproducible *in vivo* mouse model that develops primary tumors after intratibial injection within three to five weeks (24). Furthermore, this mouse model also more closely reflects human disease because it develops metastases in the lung, which is the main location of metastasis in humans (25).

We observed a reduction of 143B cell viability already after 12 h of treatment with high doses of quercetin. For 24-h treatments, the IC_{50} was 84 μM, suggesting that 143B cells are more sensitive to quercetin than HOS and MG63 cells, which showed an IC_{50} of 160 μM only after 48 h of treatment, however, they appear to be more resistant than U2-OS cells which showed an IC_{50} as low as 22 μM at 48 h of treatment (5, 6, 12).

The cell number is lower in the treated cells compared to non-treated ones 48 h after the addition of quercetin, because the cells just stopped growing. Indeed, the percentage of viable cells among those that were counted remained close to 100% at all concentrations of quercetin, except at 75 and 100 μM where viability was reduced by 20% and 40%, respectively. These results suggest that quercetin mainly exerts a cytostatic effect on 143B cells. To confirm this hypothesis, we used CFSE staining to measure cell division. In this assay, the MFI value correlates inversely with the number of cell divisions. As expected, a higher MFI signal and consequently a lower number of cell divisions were found in the quercetin-treated cells compared to the non-treated control cells. Our findings are in agreement with a study on human lung carcinoma A549 cells showing a reduction in the number of cells 24 h after quercetin treatment in a concentration-dependent manner (26). A similar result was also reported by Choi *et al*. for human breast cancer MDA-MB-453 cells, where a significant reduction in proliferation was measured with the MTT assay

after 3 h of treatment with a concentration of quercetin as low as 10 μM (27).

The cytostatic effect of quercetin can also be investigated at the level of the cell cycle. Indeed we noticed a significant increase of cells in the G_2/M phase 24 h after quercetin treatment, suggesting that in 143B cells, the inhibition of cell proliferation is directly associated with a G_2/M cell-cycle arrest. Among osteosarcoma cell lines, the only other study which carried-out cell-cycle analysis was performed with HOS cells, where an increase in G_1/S (from 66% in control to 74% in treated cells) was reported 48 h after addition of 1000 μM of quercetin (6). While this result is opposite to our findings for 143B cells, a direct comparison is invalid because the previous study used significantly higher doses of quercetin (up to 1000 μM) and a different cell line, which may result in a different response. Our findings support those of several studies carried out with other tumor cell lines. G_2/M phase arrest was reported upon quercetin treatment of human esophageal squamous carcinoma KYSE-510 cells, lung A549 and H1299 cancer cells, and human chronic myeloid K562 cells (26, 28, 29), whereas an arrest in the G_1 phase was described in breast MDA-MB-453 cancer cells and in human hepatoma HepG2 cells (27, 30). Regardless of the specific cycle phase affected (G_1/S or G_2/M) it is clear that an early effect of quercetin is cell growth arrest.

A delayed effect of quercetin is the induction of apoptosis through the mitochondrial pathway. In 143B cells, we detected PARP cleavage 24 h after treatment only at the highest concentration of quercetin used (50 μM, data not shown), whereas after 36 h, PARP cleavage was detectable in cells treated with only 25 μM quercetin. Cleaved caspase-3 and -7 were also observed at this time point. PARP cleavage-dependent apoptosis was blocked with the pan-caspase inhibitor Z-VAD. These data are in overall agreement with previous studies on other cell lines. In HOS cells, PARP cleavage was detected 48 h after the addition of 100 μM quercetin (6). In MG63 cells, mRNA expression of proteins involved in apoptosis was analyzed 48 h after the addition of different concentration of quercetin. An increase of the expression levels of the pro-apoptotic Bcl-2–associated X (BAX) protein and a reduction of the expression of the anti-apoptotic B-cell lymphoma-2 (BCL-2) were observed when cells were treated with 40 μM of quercetin (5). Cleavage of caspase-3 as well as an increase in expression of the pro-apoptotic B-cell lymphoma-extra large (BCL-XL) were also detected by western blotting in the hepatoma HepG2 cells 18 h after the addition of quercetin (31). However, in addition to the many studies suggesting an induction of mitochondrial apoptosis by quercetin, Siegelin *et al*. found that quercetin induced death receptor-mediated apoptosis in glioma cells (32). Moreover, another study performed on human colonic adenocarcinoma LS180 cells reported induction of apoptosis and necrosis after treatment with quercetin (33). Based on these studies, we conclude that the mechanism of quercetin-induced cell death is largely dependent on the cell line and in 143B osteosarcoma cells it is PARP-dependent.

Because we deliberately selected a highly metastatic osteosarcoma cell line for these studies, we also investigated *in vitro* two of the main cellular processes involved in metastasis: adhesion and migration. Migration is necessary for tumor cells to enter the lymph or blood system and later to invade new organs; adhesion is essential for the cells to settle in the newly-colonized organ. To our knowledge, no previous studies on osteosarcoma cells have addressed this issue. We now found that adhesion of 143B cells was significantly reduced in a concentration-dependent manner when they were exposed to quercetin for 12 h and adhesion was analyzed 30 min after plating the cells. Quercetin treatment also resulted in a dose-dependent reduction of cell migration when assessed in a wound-healing assay. In agreement with our findings, Lee *et al*. showed that quercetin strongly suppresses the invasive properties of MiaPaCa-2 cells *via* inhibition of matrix metalloproteinase expression (34). Moreover, Zhang *et al*. reported a quercetin concentration-dependent (20 to 80 μM) inhibition of adhesion, migration and invasion of HeLa cells (35).

Although some studies have investigated the efficacy of quercetin in osteosarcoma cells *in vitro*, our study is the first to overcome the limits of using osteosarcoma cells that are not suitable for *in vivo* studies in mice. We also demonstrated that quercetin effectively blocks some hallmarks of metastatic behavior in osteosarcoma cells. Based on these findings, we conclude that quercetin may be considered a potential drug targeting cells of the primary tumor and metastasizing foci. Further *in vivo* studies are needed to evaluate the efficacy of this compound in appropriate osteosarcoma mouse models.

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