Inhibition of nuclear factor-κB by dehydroxymethylepoxyquinomicin induces schedule-dependent chemosensitivity to anticancer drugs and enhances chemoinduced apoptosis in osteosarcoma cells

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Osteosarcoma (OS) is the most common primary malignant bone tumor, usually developing in children and adolescents, and is highly invasive and metastatic, potentially developing chemoresistance. Thus, novel effective treatment regimens are urgently needed. This study was the first to investigate the anticancer effects of dehydroxymethylepoxyquinomicin (DHMEQ), a highly specific nuclear factor-κB (NF-κB) inhibitor, on the OS cell lines HOS and MG-63. We demonstrate that NF-κB blockade by DHMEQ inhibits proliferation, decreases the mitotic index, and triggers apoptosis of OS cells. We examined the effects of combination treatment with DHMEQ and cisplatin, doxorubicin, or methotrexate, drugs commonly used in OS treatment. Using the median effect method of Chou and Talalay, we evaluated the combination indices for simultaneous and sequential treatment schedules. In all cases, combination with a chemotherapeutic drug produced a synergistic effect, even at low single-agent cytotoxic levels. When cells were treated with DHMEQ and cisplatin, a more synergistic effect was obtained using simultaneous treatment. For the doxorubicin and methotrexate combination, a more synergistic effect was achieved with sequential treatment using DHMEQ before chemotherapy. These synergistic effects were accompanied by enhancement of chemoinduced apoptosis. Interestingly, the highest apoptotic effect was reached with sequential exposure in both cell lines, independent of the chemotherapeutic agent used. Likewise, DHMEQ decreased cell invasion and migration, crucial steps for tumor progression. Our data suggest that combining DHMEQ with chemotherapeutic drugs might be useful for planning new therapeutic strategies for OS treatment, mainly in resistant and metastatic cases. *Anti-Cancer Drugs* 23:638–650 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Osteosarcoma (OS) is the most prevalent form of primary malignant bone tumor and occurs mainly in children and adolescents. Current optimal treatment consists of systemic multiagent chemotherapy and aggressive surgical resection of all sites of disease involvement. The most widely used chemotherapy treatment includes cisplatin (CDDP), doxorubicin (DX), and high-dose methotrexate (MTX) in the preoperative (induction) setting. After recovery from definitive surgery, patients receive further chemotherapy with the same three agents [1]. This treatment scheme, named adjuvant and neoadjuvant chemotherapy and adopted during the last few decades, has markedly improved the outcome of patients with localized OS (patients without evidence of metastases at diagnosis), reaching up to 65–75% overall survival rates [2,3]. However, a number of patients with nonmetastatic disease initially respond poorly (< 90% tumor necrosis) to the OS chemotherapy regimen, and consequently have a high risk of relapse and have an overall poorer outcome [4]. Moreover, it has been speculated that the sites of macroscopic disease could contain the largest reservoir of OS cells with the potential to contribute not only to metastasis but also to the development of resistance [5]. Although several clinical studies have demonstrated that inherent or acquired resistance to chemotherapeutic drugs is the most important barrier to the successful treatment of OS

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[6–8], progress in the development of antiresistance strategies remains limited.

OS patients with metastatic disease at diagnosis receive the same general treatment scheme, although the chances of long-term relapse-free survival are less than 20% [5,9]. At present, metastasis is the most important clinical variable considered when determining the risk of OS patients and is the major cause of death in OS, being radiographically detectable in 20–25% of newly diagnosed patients [10]. However, the molecular mechanisms responsible for this phenomenon are still poorly understood and the design of antimetastatic therapies is still unsatisfactory. For these reasons, drugs with new mechanisms of action and novel combination strategies are urgently needed to overcome this resistance, combat the metastatic behavior, enhance the efficacy of treatment, reduce the toxic effects of chemotherapy, and consequently improve the prognosis of OS patients.

Several studies have supported the notion that, besides its essential role in bone biology, the transcription factor nuclear factor-κB (NF-κB) signaling pathway represents a key regulator in oncogenesis [11–13]. NF-κB is a family of transcription factors present in mammalian cells and involved in the regulation of a wide variety of biological responses. This pathway is composed of five NF-κB subunits, RelA (p65), RelB, c-Rel, p50, and p52, which form homodimers and/or heterodimers. In quiescent cells, NF-κB is arrested and inactivated in the cytoplasm through interactions with inhibitory proteins of the IκB family, which comprise IκBα, IκBβ, IκBϵ, and Bcl-3, as well as the p105 and p100 precursors of p50 and p52, respectively. In a state of stimulation with a variety of signals, IκB inhibitors become phosphorylated by IκB kinases. Upon phosphorylation, IκBs undergo ubiquitination and proteosome-dependent degradation, resulting in NF-κB nuclear translocation and transcriptional activation [14]. When NF-κB is found persistently in this last state, it is referred to as constitutive activation. Inside the nucleus, NF-κB promotes the expression of several genes involved in cell proliferation [15,16], evasion of apoptosis [17], metastatic tumor spread [18], and drug resistance [19].

As constitutive activation is present in a wide variety of tumor types including OS [15,20–22] and induces the antiapoptotic machinery of tumor cells, suppression of this constitutive activation would represent a novel strategy to eliminate the oncogenic potential of these cells and makes NF-κB an interesting new therapeutic target in cancer. For these reasons, NF-κB has been newly classified as one of the major targets for drug development for cancer therapy [23]. Various NF-κB-targeting approaches have demonstrated good in-vitro or in-vivo antitumor activity in leukemias, breast cancer, prostate cancer, melanoma, lymphomas, and other tumors [23–25]. In OS, NF-κB inhibitors reduced cell proliferation [15] and TNFα-induced invasion and motility [26], and promoted osteoblastic differentiation, also causing significant malignant reversal of OS cells [15]. However, the clinical use of these gene therapy approaches is limited by delivery and stability issues.

A novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), is a derivative of the antibiotic epoxyquinomicin C [27], which has been found to specifically block TNFα-induced activation of NF-κB (mainly p65) by suppressing NF-κB nuclear translocation [28]. Besides being highly specific in nature compared with other inhibitors [29], efficiently inhibiting NF-κB DNA-binding activity and repressing transcription of NF-κB target genes, DHMEQ has shown anticancer activity in various in-vitro and in-vivo models of tumors [30–34], without observable toxicity *in vivo* [35]. However, its antitumor effects are still unknown in OS cells.

Here, we studied the antitumor effects of DHMEQ in the OS cell lines HOS and MG-63. We found that DHMEQ enhances apoptosis and significantly decreases the proliferation, invasion, and migration of OS cells. DHMEQ also acts synergistically with other chemotherapeutic drugs and this effect depends on the schedule of administration of each drug. These results demonstrate for first time the importance of DHMEQ as a promising drug for OS chemotherapy. Suitable drug administration strategies are presented and the effect of the compound in overcoming the highly metastatic behavior of this disease is described.

Materials and methods

Reagents

DHMEQ was synthesized as previously described [36], and was dissolved in dimethylsulfoxide (DMSO; Mallinckrodt Chemical Works, St Louis, Missouri, USA) at a stock concentration of 10 mg/ml. Powdered CDDP was dissolved in saline at 1.66 mmol/l and sonicated for 1 h using the method of Fischer *et al.* [37]. Both DX and MTX were diluted to 40 mmol/l stock solutions in DMSO. After preparation, all stock solutions were kept at −20°C and dissolved in culture medium immediately before the experiments. TNFα was from Calbiochem (Gibbstown, New Jersey, USA) and anti-p65 NF-κB subunit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). All reagents were purchased from Sigma, unless stated otherwise (Sigma-Aldrich, São Paulo, Brazil).

Cell culture

The human OS cell lines HOS and MG-63 were obtained from American Tissue Type Culture Collection (Rockville, Maryland, USA). The OS cell lines were maintained in HAM-F10 medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) containing

10% (v/v) fetal bovine serum (FBS; Gibco BRL, Life Technologies, Carlsbad, California, USA) at 37°C, saturated humidity, and a 5% CO_2 atmosphere. For maintenance purposes, all cell lines were passaged once or twice a week using 0.05% trypsin in 0.02% EDTA. The medium was changed twice a week under all culture conditions.

Extraction of nuclear fractions

To determine whether DHMEQ suppresses NF-κB activity by inhibiting the translocation of the NF-κB complex into the nucleus of OS cells, we designed a treatment with 10 ng/ml TNFα and/or 10 μg/ml DHMEQ alone or in combination for 3 h. Nuclear protein extracts were prepared according to the protocol of Schreiber *et al.* [38], with some modifications. Briefly, treated cells (5×10^6) were collected by trypsinization, washed twice with cold PBS, and lysed in 400 μl of cold buffer A (10 mmol/l HEPES pH 7.9, 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l ethylene glycol tetraacetic acid, 1 mmol/l phenylmethanesulfonylfluoride, 1 mmol/l dithiothreitol, 1 mg/l aprotinin, 1 mg/l leupeptin, and 1 mg/l pepstatin A). After a 15-min incubation on ice, 0.1% NP-40 was added to the homogenates and the tubes were vigorously shaken for 1 min. The homogenates were then centrifuged at 20 800*g* for 5 min at 4°C. The supernatant fluid (cytoplasmic extracts) was collected and stored in aliquots at −70°C. The nuclear pellets were washed once with cold buffer A, then suspended in 50 μl of cold buffer B (20 mmol/l HEPES, pH 7.9, 420 mmol/l NaCl, 0.1 mmol/l EDTA, 0.1 mmol/l ethylene glycol tetraacetic acid, 1 mmol/l phenylmethanesulfonylfluoride, 1 mmol/l dithiothreitol, 1 mg/l aprotinin, 1 mg/l leupeptin, and 1 mg/l pepstatin A), and vigorously shaken at maximum speed at 4°C for 30 min. The solution was clarified by centrifugation at 20 800*g* for 5 min, and the supernatant fluid (nuclear extract) was stored in aliquots at −70°C. For western blotting analysis, the protein concentration was determined by the Bradford method using an iMark microplate reader (Bio-Rad Laboratories, Hercules, California, USA).

Western blotting

Equal amounts of protein were size-fractionated by 12.0% SDS-PAGE, blotted onto an Amersham Hybond ECL nitrocellulose membrane (GE Healthcare, Piscataway, New Jersey, USA), and incubated in Tris-buffered saline – 0.1% Tween-20 containing 5% (w/v) dried nonfat milk for 1 h at room temperature. After blocking and washing in PBS with 0.1% Tween-20 for 30 min, each membrane was incubated with appropriately diluted primary antibodies at 4°C for 2 h. Following membrane incubation, the membrane was washed three times in PBS with 0.1% Tween-20 and bound to a biotin-labeled horseradish peroxidase-conjugated species-specific secondary antibody (AbCam, Cambridge, Massachusetts, USA). The complexes were visualized with an enhanced chemiluminescence reagent (ECL; Amersham, Uppsala, Sweden). The films were exposed for 30–60 s, developed, and then quantified using the ImageQuant software (Molecular Dynamics & Inc., Sunny Vale, California, USA).

In-vitro cytotoxicity

The effectiveness of DHMEQ on each cell line was assessed at 24, 48, and 72 h. The chemosensitivity to CDDP, DX, and MTX was determined at 24 h. Cells were seeded onto 96-well plates (3×10^3/well). After 24 h, the medium was replaced with fresh media containing 0–20 μg/ml of DHMEQ, 0–100 μmol/l CDDP, 0–100 μmol/l DX, or 0–100 μmol/l MTX. Proliferation inhibition was then measured by Giemsa staining of attached cells [39], with some modifications. Briefly, the culture medium was removed and the cells were washed with PBS, fixed with absolute methanol, and stained with 1% Giemsa stain. The stained cells were washed several times with tap water and the dye was dissolved with absolute methanol. The absorbance value of each well was determined at 655 nm using an iMark microplate reader (Bio-Rad, Hercules, California, USA). Each experiment was carried out using three replicates for each drug concentration and independently repeated three times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance and calculated using the Calcusyn software (Biosoft, Ferguson, Missouri, USA).

Drug combination analysis

Cell lines were treated by both simultaneous and sequential drug exposure. Subconfluent cells were exposed to 5 and 10 μmol/l CDDP, 5 and 10 μmol/l DX, and 1 μmol/l and 100 μmol/l MTX for 24 h plus 10 μg/ml DHMEQ. The simultaneous treatment regimen involved the concomitant treatment of cells with DHMEQ and either CDDP, DX, or MTX for 24 h. For sequential drug exposure, DHMEQ was added 12 h after plating and left for 12 h. The medium was aspirated, replaced with a fresh medium containing the second drug, and incubated for a further 24 h. Drug interactions were analyzed using the Calcusyn software package (BioSoft), which is based on the median effect model of Chou and Talalay [40]. A combination index (CI) score of 1 indicated an additive drug interaction, whereas a CI score more than 1 was antagonistic, and a score less than 1 was synergistic. Calcusyn software (BioSoft) was also used to calculate the dose reduction index (DRI) of drug combinations. The DRI estimates the extent to which the dose of one or more agents in the combination can be reduced to achieve effect levels that are comparable with those achieved with single agents. Drug combinations that act synergistically can be identified as those exhibiting significant dose reduction values [i.e. a given measured effect will be observed at dose(s) significantly lower than expected on the basis of single-agent activities].

Mitotic index determination

Exponentially growing cultures were treated with different concentrations of DHMEQ for 48 h at 37°C. Cells were then removed with 0.05% trypsin and complete medium was added before centrifugation at 1000 rpm for 5 min. The culture medium was discarded and the harvested cells were treated with a hypotonic potassium chloride solution (0.075 mol/l), fixed, washed in cold methanol–acetic acid, spread on glass slides, and stained with a 1% Giemsa solution. The mitotic index was determined as the percentage of mitotic cells over a total population of 1000 randomly analyzed cells. Three independent experiments were performed for each cell line.

Apoptosis assessment by annexin V/propidium iodide staining

Apoptotic cell death was determined by labeling with annexin V fluorescein isothiocyanate (BD Biosciences Pharmigen, San Jose, California, USA). Briefly, after drug treatment, 150 000 cells were trypsinized and centrifuged at 1000 rpm for 5 min at 4°C, washed with ice-cold PBS, and then resuspended in 300 µl of 1 × annexin V binding buffer (BD Biosciences Pharmigen). Cells were stained with 5 µl annexin V fluorescein isothiocyanate and 50 µl of a solution of 50 µmol/l propidium iodide, and incubated at room temperature in the dark. The samples were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences Pharmigen).

Clonogenic assay

The effects of DHMEQ on clonogenic capacity were evaluated according to Franken's protocol [41]. After trypsinization, single HOS cell suspensions of 300 cells/well were seeded in six-well plates and treated with DHMEQ at concentrations of 2.5, 5, 10, and 20 µg/ml for 24 and 48 h. After treatment, the culture medium was removed and replaced with a drug-free medium. The cell cultures were incubated for 10–15 days and the colonies were then rinsed with PBS, fixed with methanol, and stained with Giemsa stain. The colonies with more than 50 cells were counted. Assays were performed in triplicate.

Wound-healing assay for cell migration

To measure to effects of DHMEQ on cell migration, we used the Liang protocol [42], with some modifications. Briefly, 2.5×10^5 HOS cells/well were plated onto a 24-well plate in 0.1% serum medium overnight. Monolayer wounds were produced using a pipette tip scratched through the center of the well. The width of the wound was determined at time 0 (T_0). Photomicrographs of the initial wound were taken for comparison. Cells were then treated with either DMSO alone as a vehicle control, complete medium alone, 2.5, or 5 µg/ml of DHMEQ and allowed to migrate into the denuded areas for 24 h. Following incubation, cells were briefly stained with Giemsa stain. Cell migration was visualized at × 100 magnification by light microscopy and photographed using a coupled Moticam 1000 digital camera (Motic Instruments Inc., Richmond, British Columbia, Canada). The distance of migration was measured as micrometers by software Motic Images Plus 2.0 (Motic Instruments Inc.) and then compared with T_0. At least 100 different measurements per treatment were performed to obtain an average measurement. Data are the averages of triplicate determinations.

Cell invasion assay

Cell invasion was measured by the Matrigel assay using the method of Cortez *et al.* [43]. Briefly, both cell lines were treated with DHMEQ in a dose-dependent manner for 48 h in a 75 cm^2 flask. Matrigel was added to the upper surface of the transwell membrane (insert chamber) and allowed to dry in a sterile environment. After pretreatment with DHMEQ, cells were trypsinized and washed once with 1 × PBS and twice using a serum-free medium. Cell suspensions were prepared at 5×10^5 cells/ml in 0.5 ml of serum-free medium and were plated onto the upper surface of the transwell membrane. The cell-containing chamber was immersed in a lower chamber containing medium supplemented with 10% FBS. Chambers were incubated for 22 h at 37°C and 5% CO_2. Following incubation, noninvading cells were removed using a cotton-tipped swab. The cells that invaded the lower surface of the membrane were fixed in methanol and stained with Giemsa stain, digitally photographed, and counted. Each experiment was carried out in triplicate.

Statistical analysis

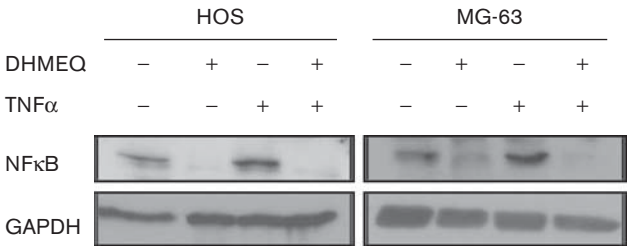
Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA), followed by nonparametric Bonferroni's Multiple Comparison test, as appropriate. All tests included comparisons with untreated samples or as indicated in the text. Statistical significance is indicated by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data analysis was carried out using the SPSS17.0 statistical software package (SPSS, Chicago, Illinois, USA). Results are reported as means ± SDs. The percentages of cell proliferation, apoptosis, survival fraction, migration, and invasion were presented graphically in the form of histograms using Microsoft Excel software (Microsoft, Redmond, Washington, USA).

Results

Dehydroxymethylepoxyquinomicin inhibits nuclear translocation of nuclear factor-κB in osteosarcoma cell lines

It has been reported that DHMEQ suppresses NF-κB activity by blocking nuclear translocation of the activated NF-κB complex into the cell nucleus [28]. We observed that in a nonstimulate state (i.e. without DHMEQ or TNFα), considerable amounts of the p65 NF-κB subunit

Fig. 1



Dehydroxymethylepoxyquinomicin (DHMEQ) suppress nuclear translocation of nuclear factor-κB (NF-κB) in osteosarcoma cells. HOS (left panels) and MG-63 (right panels) were treated with DHMEQ (10 μg/ml) or TNFα (10 ng/ml) alone or in combination in a complete medium for 3 h. Nuclear extracts from cells were subjected to analysis. Western blots of NF-κB p65 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown. GAPDH serves as an internal control.

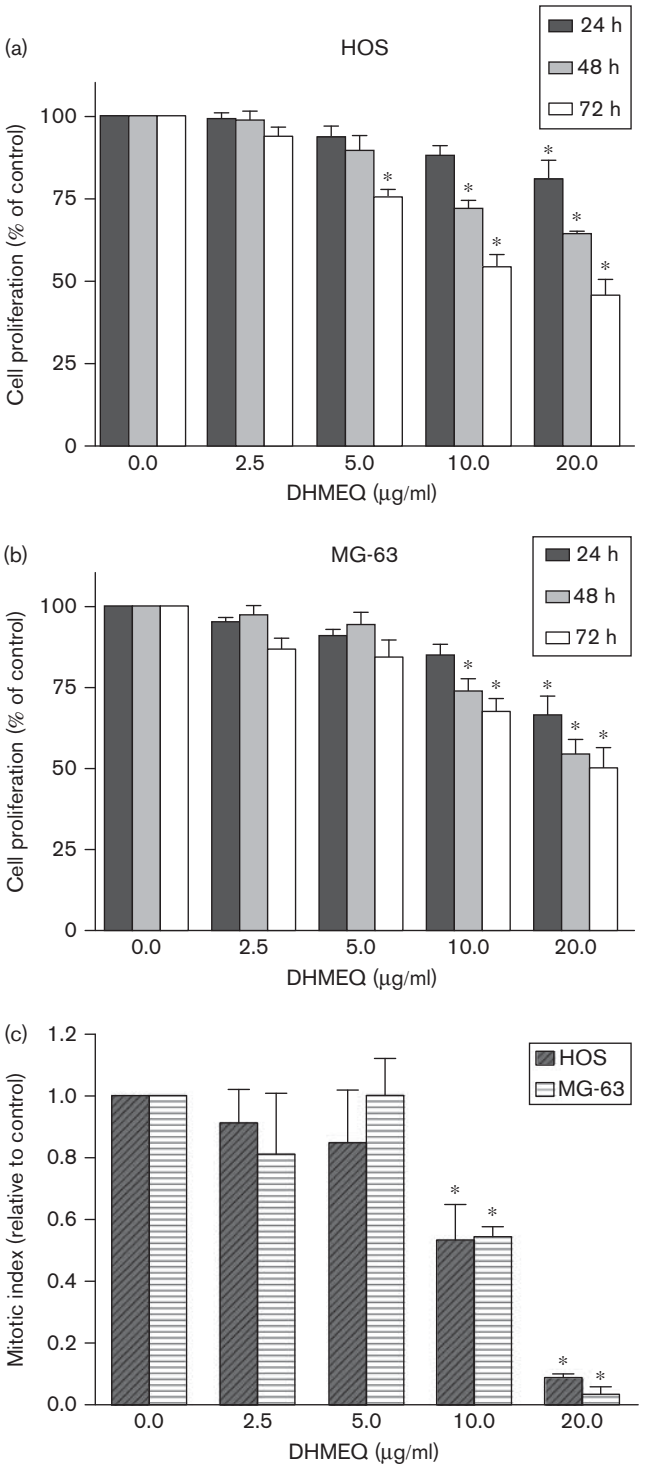
were found inside the nucleus, indicating their constitutive presence in both cell lines (Fig. 1). Cells treated with 10 μg/ml DHMEQ alone showed a decreased concentration of the p65 NF-κB subunit, demonstrating that DHMEQ effectively inhibits the translocation of NF-κB into the nucleus. As expected, incubation with 10 ng/ml TNFα greatly induced nuclear accumulation of the p65 NF-κB subunit, whereas addition of DHMEQ inhibited the nuclear translocation of NF-κB to levels comparable to those of untreated cells (Fig. 1). Finally, DHMEQ reduced both basal p65 levels and TNFα-induced accumulation of p65 in the nucleus in both tumor cell lines. These results demonstrate that OS cells have a constitutive presence of NF-κB and that DHMEQ efficiently inhibits the nuclear translocation of NF-κB in these cells.

Dehydroxymethylepoxyquinomicin inhibited the proliferation of osteosarcoma cell lines and decreased the mitotic index

Both cell cultures were sensitive to DHMEQ, showing a concentration-dependent inhibition of proliferation after the treatments ($P < 0.05$) (Fig. 2a and b). In HOS cells, a time effect difference was observed between 24 and 72 h and between 48 and 72 h ($P < 0.05$), but not between 24 and 48 h. In MG-63 cells, a time effect difference was found only between 24 and 72 h ($P < 0.05$). A significant ($P < 0.05$) inhibition was already detected after 24 h of treatment at a dose of 20 μg/ml for both cell lines. This effect became maximal after 72 h, when proliferation was reduced by more than 54 and 49% in the HOS and MG-63 cell lines, respectively.

The determination of DHMEQ-IC_{50} values revealed a remarkable sensitivity of both cell lines to DHMEQ (Table 1). For both cell lines, a significant difference was detected between the 24-h IC_{50} values (47.6 and 40.43 μg/ml, for HOS and MG-63, respectively) and 48-h

Fig. 2



Dehydroxymethylepoxyquinomicin (DHMEQ) inhibits cell proliferation and the mitotic index (MI) in osteosarcoma (OS) cells. Cell proliferation in (a) HOS and (b) MG-63 following incubation with serial doses of DHMEQ for 24, 48, and 72 h. After treatments, the inhibition of cell proliferation was determined using a Giemsa-staining assay. (c) MI in OS cells treated with serial doses of DHMEQ. A total of 1000 cells were scored in each experiment. Data represent mean ± SD of three independent experiments. *$P < 0.05$.

IC_{50} values (18.45 and 19.86 μg/ml for HOS and MG-63; $P < 0.05$). The same difference was observed between the 24- and 72-h IC_{50} values (12.9 and 19.1 μg/ml, for HOS and MG-63, respectively; $P < 0.05$). No significant difference was found between the 48- and 72-h IC_{50} values (Table 1).

As shown in Fig. 1c, DHMEQ also caused a significant decrease in the mitotic index starting at the 10 μg/ml DHMEQ treatment in both cell lines. The most significant reduction was achieved with 20 μg/ml DHMEQ, when only 8.7 and 3.2% mitotic cells were counted in the HOS and MG-63 lines, respectively.

Our results demonstrated that DHMEQ produced efficient growth inhibition and a marked decrease in mitotic activity in OS cells.

Osteosarcoma cells lines were sensitive to cisplatin and doxorubicin, but not to methotrexate

In chemosensitivity studies, dose–response curves and IC_{50} values were calculated for CDDP, DX, and MTX at 24 h. The comparison between CDDP, DX, and MTX IC_{50} values confirmed that both cell lines were sensitive to CDDP and DX, but not to MTX. As shown in Table 1, HOS cells ($IC_{50} = 44.26$ μmol/l) proved to be more sensitive to CDDP than MG-63 cells ($IC_{50} = 80.28$ μmol/l). The same was observed for DX, whose IC_{50} values were 78.89 μmol/l versus 280.89 μmol/l (HOS vs. MG-63). In MTX analysis, no IC_{50} values were detected for either cell line at concentrations up to 100 μmol/l. Thus, we considered these cells to be resistant to MTX. Our results confirm that both cell lines were sensitive to CDDP and DX, but not for MTX.

Dehydroxymethylepoxyquinomicin acts synergistically with chemotherapeutic drugs

To determine whether DHMEQ sensitizes OS cell lines to conventional chemotherapy drugs, we measured the effects of combined treatments and determined the best treatment schedule with either simultaneous or sequential drug exposure. As the cell lines were sensitive to CDDP and DX, but not MTX, and considering that low concentrations of chemotherapy are most practical for clinical use, we aimed to use the lowest possible dose in these experiments. In this context, cells were exposed to 10 μg/ml DHMEQ and to low concentrations (5 and 10 μmol/l) of either CDDP or DX; and for the MTX combinations, a low dose and a high dose (1 and 100 μmol/l) were used in the experiments.

As shown in Table 2, DHMEQ acted synergistically ($CI < 1$) with each conventional drug in both cell lines in a treatment schedule-independent manner. Determination of the best administration schedule revealed different drug-dependent behaviors (responses). For CDDP combinations, we found that simultaneous exposure to DHMEQ and CDDP (DHMEQ + CDDP) had a greater inhibitory effect than sequential exposure in both cell lines. The synergistic effect of simultaneous drug exposure was

Table 1 Doses required to induce 50% inhibition of cell growth (IC_{50}) in osteosarcoma cell lines

Cell line	DHMEQ 24 h (μg/ml)	DHMEQ 48 h (μg/ml)	DHMEQ 72 h (μg/ml)	CDDP 24 h (μmol/l)	DX 24 h (μmol/l)	MTX 24 h (μmol/l)
HOS	47.6 ± 9.23	18.45 ± 4.14	12.9 ± 1.14	44.26 ± 5.23	78.89 ± 4.38	>100
MG-63	40.43 ± 5.25	19.86 ± 1.27	19.10 ± 5.51	80.28 ± 6.74	280.89 ± 11.6	>100

Data represent mean ± SD of three independent experiments.
CDDP, cisplatin; DHMEQ, dehydroxymethylepoxyquinomicin; DX, doxorubicin; MTX, methotrexate.

Table 2 Fa, CI, and DRI values for DHMEQ combinations with CDDP, DX, and MTX

Cell line	HOS			MG-63		
Combined effects of DHMEQ with:	Fa	CI	DRI to chemotherapy drug	Fa	CI	DRI to chemotherapy drug
+ 5 μmol/l CDDP	0.5797	0.177	43.8	0.304322	0.653	7.743
+ 10 μmol/l CDDP	0.6644	0.126	138.160	0.437626	0.461	6.435
/5 μmol/l CDDP	0.5448	0.217	21.637	0.275123	0.745	6.831
/10 μmol/l CDDP	0.322279	9.440	0.110	0.162679	1.647	1.892
+ 5 μmol/l DX	0.641956	0.156	34.822	0.331917	0.513	20.786
+ 10 μmol/l DX	0.67112	0.164	20.750	0.462174	0.323	22.645
/5 μmol/l DX	0.7571	0.098	73.725	0.43392	0.336	38.499
/10 μmol/l DX	0.6714	0.164	20.790	0.393949	0.428	15.228
+ 1 μmol/l MTX	0.3157	0.346	5.28×10^{9}	0.285054	0.571	5.33×10^{5}
+ 100 μmol/l MTX	0.394523	0.268	7.11×10^{16}	0.330138	0.469	2.1×10^{4}
/1 μmol/l MTX	0.4929	0.200	2.78×10^{29}	0.395053	0.361	1.3×10^{7}
/100 μmol/l MTX	0.5565	0.165	1.57×10^{34}	0.452061	0.290	5.92×10^{5}

Cells were treated with 10 μmol/l DHMEQ and either simultaneously (+) or sequentially (/) drug chemotherapeutic treatment. Dose reduction index (DRI) reflects the fold reduction in the required concentration of tested agents when used in combination to achieve the fraction affected (Fa). The combination index (CI) correlates the cytotoxicity of drug combinations to the single-agent activities. Interpretation of CI values: less than 0.1 = very strong synergism, 0.1–0.3 = strong synergism, 0.3–0.7 = synergism, 0.7 to 0.9 = slight synergism, 0.9–1.1 = additive effect, and more than 1.1 = antagonism.
CDDP, cisplatin; DHMEQ, dehydroxymethylepoxyquinomicin; DX, doxorubicin; MTX, methotrexate.

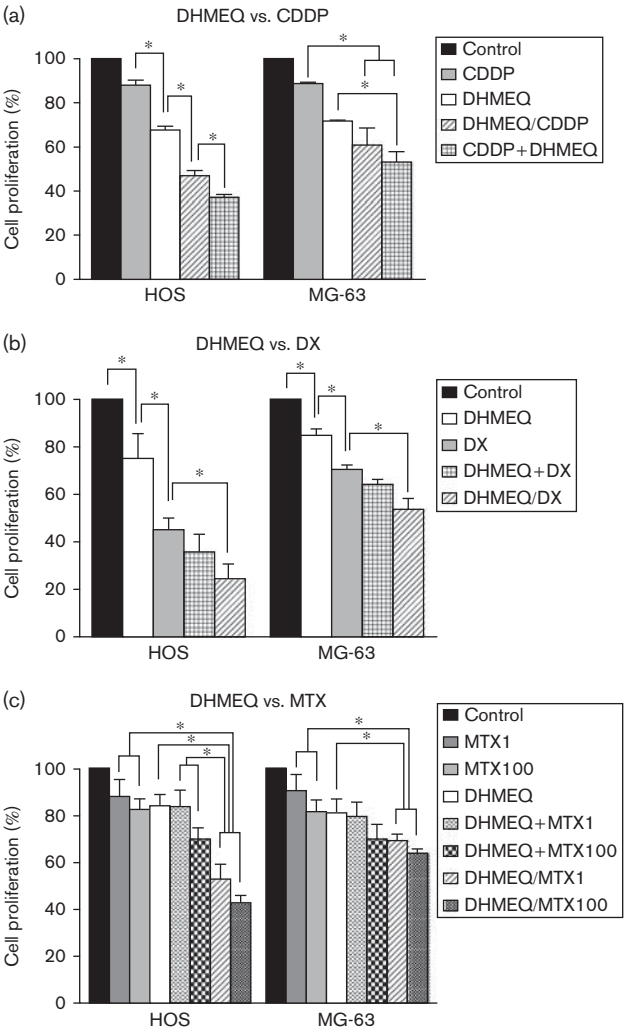
higher in the HOS than in the MG-63 cell line. In HOS cells, both simultaneous combined CDDP doses (5 and 10 μmol/l CDDP) resulted in strong synergism (CIs = 0.177 and 0.126, respectively) and produced a very high DRI (43.8 and 138.16, respectively) when compared with the MG-63 cell line (Table 2). Similarly, sequential DHMEQ/CDDP administration produced a synergistic effect at 5 μmol/l CDDP. However, when the dose of CDDP was increased to 10 μmol/l CDDP, an antagonistic effect was observed in both cell lines and was more intense in HOS cells (CI = 9.44) than in MG-63 cells (1.647). ANOVA revealed a significant difference between CDDP alone (5 μmol/l), DHMEQ alone (10 μg/ml), and the respective combined treatments (Fig. 3a).

In the case of DX, all the combinations performed showed synergism. For both cell lines, the highest synergistic effect was achieved with sequential combinations (DHMEQ/DX) using 5 μmol/l concentrations, and the CI values obtained with this combination were 0.098 and 0.336, for HOS and MG-63, respectively. Consequently, the DRI values obtained with this drug combination were the highest for this drug, 73.7 and 38.5 for HOS and MG-63 cells, respectively. In both cell lines, the difference in growth inhibition between DHMEQ alone (10 μg/ml), DX alone (5 μmol/l), and the DHMEQ/DX exposure was found to be statistically significant ($P < 0.05$) (Fig. 3b).

A synergistic effect was also found in all combinations of DHMEQ and MTX in OS cells. The most synergistic combination was obtained by sequential administration, and the lowest CIs were 0.165 and 0.290 for HOS and MG-63 cells, respectively. These CI were obtained with sequential DHMEQ/MTX treatment (10 μg/ml DHMEQ followed by 100 μmol/l MTX), causing a strong synergism in both cell lines (Table 2). As shown in Fig. 3c, we confirmed by ANOVA the differences in growth inhibition between MTX alone, DHMEQ alone, and DHMEQ/MTX treatments in OS cells ($P < 0.05$).

Independent of the drug administration schedule, our results showed that DHMEQ (10 μg/ml) acted synergistically with CDDP (5 μmol/l), DX (5 or 10 μmol/l), and MTX (1 or 100 μmol/l), drugs commonly used in OS treatment.

Fig. 3



Dehydroxymethylepoxyquinomicin (DHMEQ) acts synergistically with chemotherapeutic drugs. Proliferation assays were performed using both osteosarcoma cell lines, incubated with 10 μg/ml DHMEQ; (a) 5 μmol/l cisplatin (CDDP); (b) 5 μmol/l doxorubicin (DX); (c) 1 or 100 μmol/l methotrexate (MTX1 or MTX100, respectively), and either simultaneously (+) or sequentially (/) drug combination treatment for a total time exposure of 24 h. After treatments, the inhibition of proliferation was determined using a Giemsa-staining assay. The values were plotted as a percentage of control cells treated with vehicle alone. Data represent mean ± SD of three independent experiments. *$P < 0.05$.

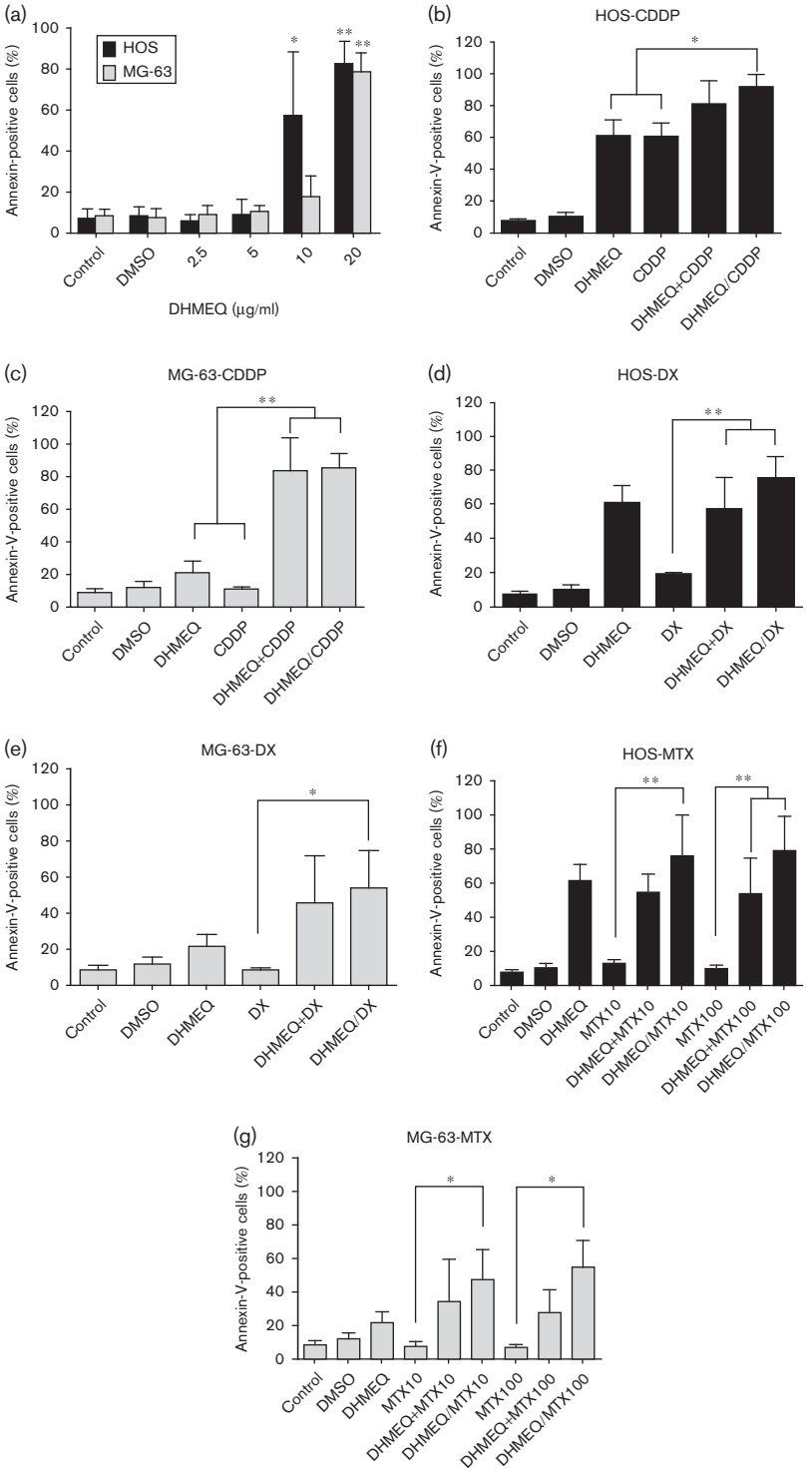
Dehydroxymethylepoxyquinomicin causes and enhances apoptotic cell death in osteosarcoma cells

To determine whether DHMEQ induced apoptosis in OS cells, we used the annexin V/propidium iodide assay. As indicated in Fig. 4a, the control cells, as expected, were viable, whereas the number of apoptotic cells increased in treated cells (annexin-V-positive cells). Statistical analysis showed a significant difference starting at 10 and 20 μg/ml for HOS and MG-63, respectively (Fig. 4a). These results suggest that DHMEQ induces apoptosis in HOS and MG-63 cells.

Given the significant synergistic effect found between DHMEQ and chemotherapeutic drugs (CDDP, DX, and MTX) in proliferation assays, and the better drug administration schedule found for HOS and MG-63 cells, we evaluated apoptosis under the same treatment conditions for both cell lines.

As shown in Fig. 4b–g, combined treatments with DHMEQ and each chemotherapeutic drug significantly increased the percentage of apoptotic cells when compared with each drug alone in both cell lines. In all cases, the highest enhancement of apoptosis was

Fig. 4



Dehydroxymethylepoxyquinomicin (DHMEQ) causes apoptosis and enhances chemoinduced apoptosis in osteosarcoma cells. (a) DHMEQ-treated OS cells for 48 h. The percentage of annexin-V-positive cells of a cell count of 10 000 events was obtained. Data represent mean ± SD of three independent experiments. $*P<0.05$, $**P<0.01$ compared with vehicle-alone treatment. (b–g) DHMEQ enhanced chemoinduced apoptosis on OS cells. HOS and MG-63 cells were treated with 10 μg/ml DHMEQ, (b–c) 5 μmol/l cisplatin (CDDP); (d–e) 5 μmol/l doxorubicin (DX); (f–g) 10 or 100 μmol/l methotrexate (MTX10 or MTX100, respectively), and either simultaneously (+) or sequentially (/) drug combination treatment for a total time exposure of 24 h. In all cases, viable control cell (control) and vehicle alone treatment [dimethylsulfoxide (DMSO)] were used. Analysis was performed by flow cytometry. Percentage of annexin-V-positive cells is showed. Data represent mean ± SD of three independent experiments. $*P<0.05$; $**P<0.01$ compared with DHMEQ, CDDP, DX, MTX10, or MTX100 alone treatment.

Table 3 Effect of dehydroxymethylepoxyquinomicin in chemoinduced apoptosis

	Enhancement apoptosis by drug treatment			
Cell line	5 μmol/l CDDP	5 μmol/l DX	10 μmol/l MTX	100 μmol/l MTX
Sequential administration effect/chemotherapy drug alone effect				
HOS	1.52 times	3.87 times	5.89 times	8.03 times
MG-63	7.82 times	6.45 times	6.35 times	8.09 times
Simultaneous administration effect/chemotherapy drug alone effect				
HOS	1.35 times	2.96 times	4.25 times	5.48 times
MG-63	7.67 times	5.46 times	4.59 times	4.05 times

Values represent the ratio of the number of apoptotic cells (annexin-V-positive cells) by sequential or simultaneous treatment versus the number of apoptotic cell by chemotherapeutic drug alone treatment in each cell line.
CDDP, cisplatin; DX, doxorubicin; MTX, methotrexate.

achieved with the sequential administration of DHMEQ and after CDDP (Fig. 4b–c), DX (Fig. 4d–e), or MTX (Fig. 4f–g). To quantify the enhancement of apoptosis caused by DHMEQ, we calculated the ratio of the number of apoptotic cells obtained by sequential or simultaneous treatment versus the number of apoptotic cell obtained by treatment with each chemotherapeutic drug alone in each cell line (Table 3). The most intense effect was observed in the MG-63 cell line after sequential treatment with 5 μmol/l CDDP, 5 μmol/l DX, 10 μmol/l MTX, or 100 μmol/l, when apoptosis was enhanced 7.82, 6.45, 6.35, and 8.09 times compared with the effect of each drug alone, respectively.

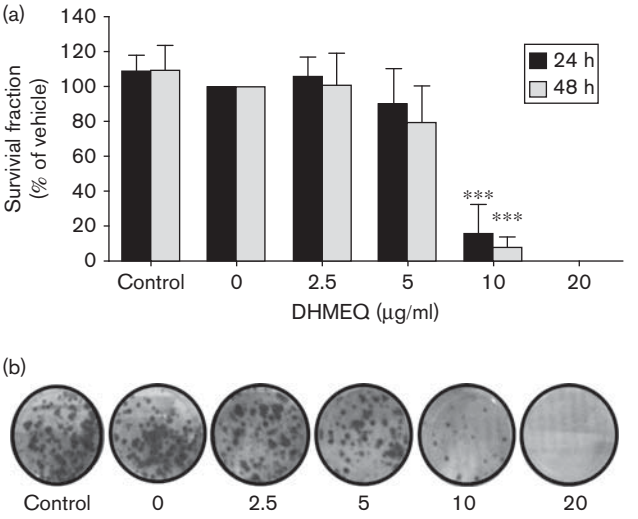
Dehydroxymethylepoxyquinomicin inhibited colony formation in HOS cells

After the treatments and following incubation, colonies with a minimum of 50 cells were counted and the surviving fraction was determined. The efficiency of plating was 33.48 ± 3.7% for 24 h and 41.74 ± 6.7% for 48 h. DHMEQ inhibited the colony formation of HOS cells, reaching a significant difference starting at 10 μg/ml ($P < 0.05$) (Fig. 5). At 20 μg/ml, no colonies were formed.

Dehydroxymethylepoxyquinomicin impaired the migration and invasion of HOS cells

HOS cells were plated in 0.1% serum medium overnight before inducing the wound to ensure that migration rather than cell growth was measured. As DHMEQ concentrations higher than 5 μg/ml caused cell detachment, we treated the cells with 2.5 or 5 μg/ml DHMEQ, DMSO alone, or only 0.1% serum medium for 24 h. Representative results are shown for HOS cells, which were digitally photographed, and the width of the denuded area in the wound was measured in micrometers (Fig. 6a). Wound healing was significantly inhibited by DHMEQ at 5 μg/ml ($P < 0.05$) (Fig. 6b). To evaluate the effect of DHMEQ on cell invasion, we performed the Matrigel assay. HOS and MG-63 cells were treated with DHMEQ in a dose-dependent manner for 48 h. Cells were then added to Matrigel invasion chambers and incubated for 22 h. Following incubation, cells that invaded the lower surface

Fig. 5



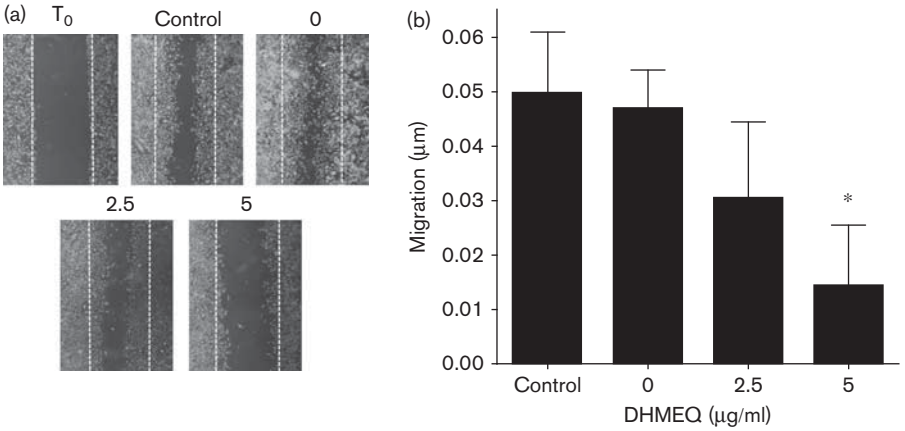
Dehydroxymethylepoxyquinomicin (DHMEQ) decreases the clonogenic survival of osteosarcoma cells. (a) HOS cells were treated for 24 and 48 h. Untreated cells and cells treated with vehicle alone (dimethylsulfoxide, DMSO) served as controls. The colonies formed were normalized to the plating efficiency and compared with DMSO treatment. DHMEQ inhibited significantly clonogenic capacity at 10 μmol/l, and no colony was formed at 20 μmol/l. Data represent mean ± SD of three independent experiments. ***$P < 0.001$.
(b) Representative photography of DHMEQ's effect on HOS clonogenic survival is shown.

of the membrane were fixed, stained, photographed, and counted. Each experiment was carried out in triplicate. We found significant 36 and 24% reductions in invasion compared with the control when using 10 μg/ml DHMEQ, in HOS and MG-63 cells, respectively (Fig. 7). Although a greater inhibition of invasion was observed in HOS cells, a significant reduction was observed in the MG-63 cell line ($P < 0.05$) with the use of a lower dose (5 μg/ml) (Fig. 7b).

Discussion and conclusion

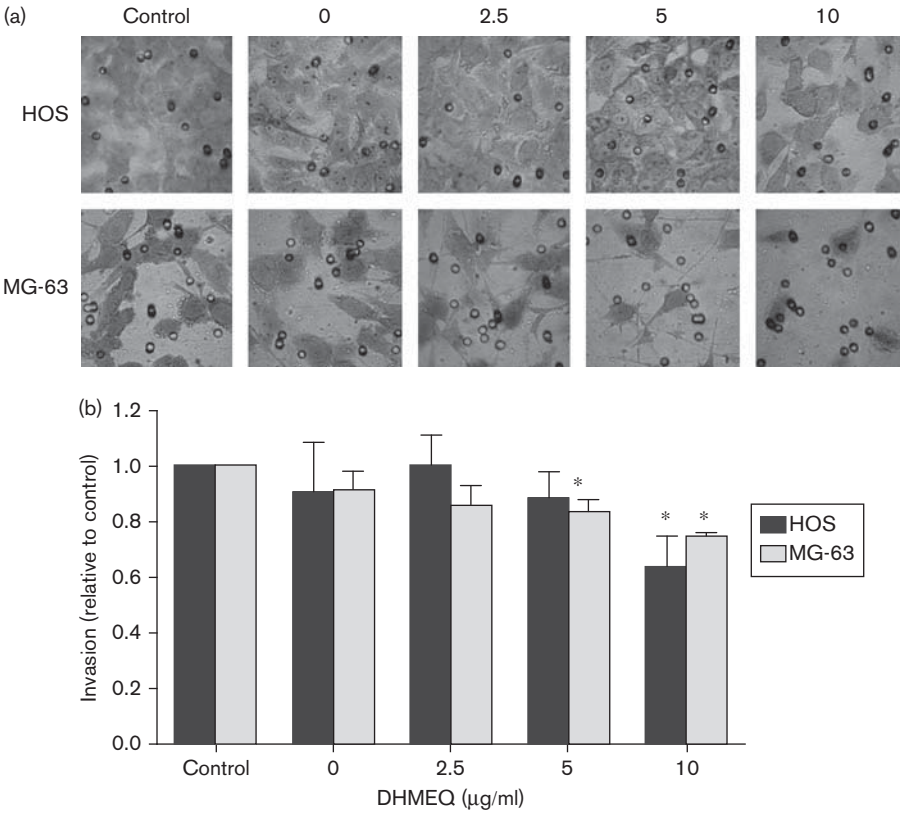
Although the 5-year survival rate of metastatic OS patients has remained at 20% for the last 15 years, the same chemotherapeutic regimen continues to be used. Metastasis is considered to be the most important clinical variable when assigning risk to OS patients and represents the major cause of death in OS. However, the success of new antiprogression and antimetastatic behavior strategies remains unsatisfactory. Here, we report for the first time an investigation of NF-κB inhibition by DHMEQ in OS cells. We found that DHMEQ inhibited cell proliferation and chemosensitized OS cells to conventional drugs. Moreover, our study also suggests an efficient schedule of drug administration. We further demonstrated that DHMEQ alone triggered apoptosis in a dose-dependent manner and enhanced apoptosis when combined with chemotherapeutic drugs. DHMEQ also showed important antiprogression effects such as inhibition of migration and invasion in these cells.

Fig. 6



Dehydroxymethylepoxyquinomicin (DHMEQ) inhibits migration of osteosarcoma (OS) cells. Wound-healing assays were performed in HOS cells. (a) Cell monolayers starved overnight (0.1% FBS) were used to perform wounds before the DHMEQ treatment (2.5 or 5 μg/ml) for 24 h. The dose of 5 μmol/l DHMEQ was able to reduce cell migration of HOS cells significantly. (b) Width of voided area versus DHMEQ dose concentration was graphed to express the degree of inhibition of cell migration. Bars represent mean ± SD of three independent experiments. *$P < 0.05$.

Fig. 7



Dehydroxymethylepoxyquinomicin (DHMEQ) inhibits the invasion of osteosarcoma cells. An invasion assay using transwell chambers coated with Matrigel was performed in HOS and MG-63 cells, 48 h after DHMEQ treatment. (b) The mean values of invasive cells were graphed versus DHMEQ concentration. Both cell lines presented a significant decrease in the invasion rate after DHMEQ treatment, compared with the control. The decrease in the invasion rate was observed since 5 μmol/l for MG-63 cells and 10 μmol/l for HOS cells. Data represent mean ± SD of three independent experiments. *$P < 0.05$.

DHMEQ exhibits antitumor activity by blocking the translocation of NF-κB p65 into the nucleus, a unique mechanism of action when compared with other NF-κB inhibitors. In our study, western blotting assays showed that DHMEQ effectively inhibits both constitutive and TNFα-mediated nuclear translocation of NF-κB-containing p65 in OS cells.

When the antiproliferative activity was assessed, DHMEQ treatment caused a significant inhibition in proliferation, showing that activation of NF-κB plays an important role in the growth of these cells. This hypothesis has been supported by investigations including various tumor models such as synovial sarcoma [44], cancer of the bladder [45] and of the thyroid [31], multiple myeloma [32], breast cancer [33], T-cell leukemia [35], prostate cancer [30], and head and neck cancer [34], in which similar concentrations of DHMEQ were used and equivalent effects were obtained. Thus, we may assume that the growth of OS cells is largely dependent on a marked activity of NF-κB signaling.

Furthermore, studies have demonstrated that NF-κB blockade causes apoptosis induction and cell cycle arrest in OS cells [15,46,47]. In our study, DHMEQ triggered apoptosis in both OS cell lines, mainly in HOS cells, efficiently reduced mitotic activity, and markedly affected the colony-forming capacity. These results demonstrate the efficient anticancer activity of DHMEQ in OS cells and suggest that the effects of DHMEQ may depend on the downregulation of NF-κB target genes involved in growth, and control apoptosis, cell cycle, and OS progression, as also proposed in other studies [29].

The prosurvival activity of NF-κB induced by traditional chemotherapeutic drugs is crucial for chemoresistance in cancer [44,48,49]. As chemoresistance is the most important barrier for successful OS treatment, we hypothesized that NF-κB inhibition by DHMEQ could chemo-sensitize and circumvent the mechanism of tumor resistance in OS.

Our study confirmed this hypothesis because DHMEQ acted synergistically with all the most important drugs currently used in OS chemotherapy (CDDP, DX, and MTX). Of particular interest was also the evidence that all the synergistic effects found in our study were obtained from drug combinations using suboptimal chemotherapy concentrations. These data suggest that DHMEQ, when used for OS treatment, would not only improve the clinical outcome but also induce minimal or no adverse side effects.

All combinations of DHMEQ with chemotherapeutic drugs were more synergistic in HOS than in MG-63 cells. We speculate that the greater sensitivity of HOS cells to drugs occurs because these cells have a high proliferation rate compared with MG-63 cells, as demonstrated by comparison of doubling times values between the two cell lines [50].

To find the most efficient schedule of drug administration, we assessed the interactions obtained between DHMEQ and conventional drugs in OS cell lines. Simultaneous exposure to DHMEQ and CDDP (DHMEQ + CDDP) was more efficient (lower CI) than sequential exposure (DHMEQ/CDDP) for both cell lines. We hypothesized that sequential exposure (DHMEQ/CDDP) would cause many molecular modifications in the OS cell. Initially, DHMEQ exposure causes antiproliferation effects that are suppressed after DHMEQ privation. After CDDP addition, new prosurvival molecular events emerge, as demonstrated by Poma *et al.* [51]. In this case, initial treatment with CDDP alone upregulated interleukin 6 (IL-6). This upregulation was decreased by treatment with CDDP plus DHMEQ. IL-6 is an important inflammatory cytokine involved in cancer proliferation owing to its ability to activate NF-κB [52]. We think that in simultaneous treatment (DHMEQ + CDDP), the continuous activity of DHMEQ would be able to maintain inhibition of any possibility of CDDP-mediated resistance. Studies to determine the involvement of IL-6 and/or other factors in OS chemoresistance would be enlightening.

For DX and MTX, we obtained synergy with both drug administration schedules (sequential and simultaneous exposure) in both cell lines. Although various studies have shown a synergistic effect between DHMEQ and various chemotherapeutic agents, including DX [44,53–55], no studies have evaluated the effects of combined DHMEQ and MTX. Our study is the first report demonstrating that DHMEQ acts synergistically with MTX. Although both cell lines were resistant to MTX treatment alone, combinations of DHMEQ and MTX resulted in the lowest CIs values. Regarding the more efficient administration schedule, we found that sequential exposure (DHMEQ/DX or DHMEQ/MTX) exerted a more synergistic effect than simultaneous exposure in both cell lines.

The mechanisms of action of these agents through different growth-signaling pathways suggest the potential for the clinical use of subtherapeutic doses of CDDP, DX, and mainly MTX in combination with DHMEQ, which will allow effective suppression of tumor growth while minimizing the toxic side effects of these drugs, and overcome the barrier of chemoresistance. Besides, according to our results, we suggest that the most efficient drug administration schedule for OS treatment is simultaneous exposure to DHMEQ and CDDP, and sequential exposure to DHMEQ and DX or to DHMEQ and MTX.

Sequential administration (DHMEQ/chemotherapeutic drug) sensitized cells to CDDP-induced, DX-induced, and MTX-induced apoptosis. This result confirms the

potential of DHMEQ to enhance apoptosis and/or to reverse apoptosis resistance in OS cells, corroborating the 'two signal model' hypothesis [56]. Thus, DHMEQ disarms the antiapoptotic machinery governed by the NF-κB pathway and, in a second stage, permits CDDP, DX, or MTX to carry out both their cytotoxic and apoptotic functions.

OS is highly metastatic and invasive and the patients with metastases at diagnosis rarely achieve a good survival rate. In our study, DHMEQ was able to significantly decrease cell migration and invasion, two necessary steps for metastasis development. Few studies have explored the antiinvasion and antimigration capacity of DHMEQ [29,57]. On the basis of our results, we believe that DHMEQ may be a promising agent against the high metastatic behavior of OS.

Finally, we consider our in-vitro model to be sufficiently useful to obtain crucial evidence about the anticancer properties of DHMEQ. In addition, it was possible to propose a suitable schedule for drug administration using suboptimal doses of each chemotherapeutic agent. Our study also showed that DHMEQ is able to trigger apoptosis, enhance chemoinduced apoptosis, and inhibit cell migration and invasion in both OS cell lines. Additional in-vivo evaluation, as well as investigation of the molecular consequences after drug administration would be enlightening. The present study indicates the potential usefulness of DHMEQ in novel treatment strategies for human OS, mainly focusing on chemoresistant cases and metastatic disease.

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Conflicts of interest

There are no conflicts of interest.

References

1 Chou AJ, Geller DS, Gorlick R. Therapy for osteosarcoma: where do we go from here? *Paediatr Drugs* 2008; **10**:315–327.
2 Meyers PA, Heller G, Healey J. Chemotherapy for nonmetastatic osteogenic sarcoma: the Memorial Sloan-Kettering experience. *J Clin Oncol* 1992; **10**:5–15.
3 George SL. Response rate as an endpoint in clinical trials. *J Natl Cancer Inst* 2007; **99**:98–99.
4 Walters DK, Steinmann P, Langsam B, Schmutz S, Born W, Fuchs B. Identification of potential chemoresistance genes in osteosarcoma. *Anticancer Res* 2008; **28**:673–680.
5 Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: currents challenges and future directions. *Expert Rev Anticancer Ther* 2006; **6**:1075–1085.
6 Davis AM, Bell RS, Goodwin PJ. Prognostic factors in osteosarcoma: a critical review. *J Clin Oncol* 1994; **12**:423–431.
7 Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, *et al.* Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol* 2002; **20**:776–790.
8 Bacci G, Ferrari S, Bertoni F, Picci P, Bacchini P, Longhi A, *et al.* Histologic response of highgrade nonmetastatic osteosarcoma of the extremity to chemotherapy. *Clin Orthop* 2001; **386**:186–196.
9 Lamoureux F, Trichet V, Chipoy C, Blanchard F, Gouin F, Redini F. Recent advances in the management of osteosarcoma and forthcoming therapeutic strategies. *Expert Rev Anticancer Ther* 2007; **7**:169–181.
10 Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. *Oncologist* 2004; **9**:422–441.
11 Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002; **2**:301–310.
12 Kim HJ, Hawke N, Baldwin AS. NF-kappaB and IKK as therapeutic targets in cancer. *Cell Death Differ* 2006; **13**:738–747.
13 Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 1999; **18**:6938–6947.
14 Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 2000; **18**:621–663.
15 Andela VB, Sheu TJ, Puzas JE, Schwarz EM, O'Keefe RJ, Rosier RN. Malignant reversion of a human osteosarcoma cell line, Saos-2, by inhibition of NFκB. *Bioch Biophys Res Commun* 2002; **297**:237–241.
16 Eliseev RA, Schwarz EM, Zuscik MJ, O'Keefe RJ, Drissi H, Rosier RN. Smad7 mediates inhibition of Saos2 osteosarcoma cell differentiation by NFκB. *Exp Cell Res* 2006; **312**:40–50.
17 Millet I, Phillips RJ, Sherwin RS, Ghosh S, Voll RE, Flavell RA, *et al.* Inhibition of NF-κB activity and enhancement of apoptosis by the neuropeptide calcitonin generelated peptide. *J Biol Chem* 2000; **275**:15114–15121.
18 Andela VB, Schwarz EM, Puzas JE, O'Keefe RJ, Rosier RN. Tumor metastasis and the reciprocal regulation of prometastatic and antimetastatic factors by nuclear factor KappaB. *Cancer Res* 2000; **60**:6557–6562.
19 Bentires-Alj M, Barbu V, Fillet M, Chariot A, Relic B, Jacobs N, *et al.* NF-kappaB transcription factor induces drug resistance through MDR1 expression in cancer cells. *Oncogene* 2003; **22**:90–97.
20 Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, *et al.* Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997; **100**:2961–2969.
21 Kordes U, Krappmann D, Heissmeyer V, Ludwig WD, Scheidereit C. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. *Leukemia* 2000; **14**:399–402.
22 Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells in inhibited by ibuprofen. *Oncogene* 1999; **18**:7389–7394.
23 Lee CH, Jeon YT, Kim SH, Song YS. NF-κB as a potential molecular target for cancer therapy. *BioFactors* 2007; **29**:19–35.
24 Huang S, Pettaway CA, Uehara H, Bucana CD, Fidler IJ. Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene* 2001; **20**:4188–4197.
25 Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF-kB activity and apoptosis in chronic lymphocytic leukemia B cells. *J Immunol* 2000; **164**:2200–2206.
26 Harimaya K, Tanaka K, Matsumoto Y, Sato H, Matsuda S, Iwamoto Y. Antioxidants inhibit TNF-α-induced motility and invasion of human osteosarcoma cells: possible involvement of NFκB activation. *Clin Exp Metast* 2000; **18**:121–129.
27 Matsumoto N, Ariga A, To-e S, Nakamura H, Agata N, Hirano S, *et al.* Synthesis of NF-kappaB activation inhibitors derived from epoxyquinomicin C. *Bioorg Med Chem Lett* 2000; **10**:865–869.
28 Ariga A, Namekawa J, Matsumoto N, Inoue J, Umezawa K. Inhibition of tumor necrosis factor-alpha-induced nuclear translocation and activation of NF-kappa B by dehydroxymethylepoxyquinomicin. *J Biol Chem* 2002; **277**:24625–24630.
29 Wong JHT, Lui VWY, Umezawa K, Ho Y, Wong EYL, Ng MHL, *et al.* A small molecule inhibitor of NF-κB, dehydroxymethylepoxyquinomicin (DHMEQ),

suppresses growth and invasion of nasopharyngeal carcinoma (NPC) cells. *Cancer Lett* 2010; **287**:23–32.
30 Kikuchi E, Horiguchi Y, Nakashima J, Kuroda K, Oya M, Ohigashi T, *et al*. Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. *Cancer Res* 2003; **63**:107–110.
31 Starenki DV, Namba H, Saenko VA, Ohtsuru A, Maeda S, Umezawa K, *et al*. Induction of thyroid cancer cell apoptosis by a novel nuclear factor kappaB inhibitor, dehydroxymethylepoxyquinomicin. *Clin Cancer Res* 2004; **10**:6821–6829.
32 Watanabe M, Dewan MZ, Okamura T, Sasaki M, Itoh K, Higashihara M, *et al*. A novel NF-kappaB inhibitor DHMEQ selectively targets constitutive NF-kappaB activity and induces apoptosis of multiple myeloma cells in vitro and in vivo. *Int J Cancer* 2005; **114**:32–38.
33 Matsumoto G, Namekawa J, Muta M, Nakamura T, Bando H, Tohyama K, *et al*. Targeting of nuclear factor kappaB pathways by dehydroxymethylepoxyquinomicin, a novel inhibitor of breast carcinomas: antitumor and antiangiogenic potential in vivo. *Clin Cancer Res* 2005; **11**:1287–1293.
34 Ruan HY, Masuda M, Ito A, Umezawa K, Nakashima T, Yasumatsu R, *et al*. Effects of a novel NF-kappaB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), on growth, apoptosis, gene expression, and chemosensitivity in head and neck squamous cell carcinoma cell lines. *Head Neck* 2006; **28**:158–165.
35 Watanabe M, Ohsugi T, Shoda M, Ishida T, Aizawa S, Maruyama-Nagai M, *et al*. Dual targeting of transformed and untransformed HTLV-1-infected T cells by DHMEQ, a potent and selective inhibitor of NF-kappaB, as a strategy for chemoprevention and therapy of adult T-cell leukemia. *Blood* 2005; **106**:2462–2471.
36 Umezawa K, Chaicharoenpong C. Molecular design and biological activities of NF-kappaB inhibitors. *Mol Cells* 2002; **14**:163–167.
37 Fischer SJ, Benson LM, Fauq A, Naylor S, Windebank AJ. Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. *Neurotoxicology* 2008; **29**:444–452.
38 Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 1989; **17**:6419.
39 Galfi P, Neogrady Z, Amberger A, Margreiter R, Csordas A. Sensitization of colon cancer cell lines to butyrate-mediated proliferation inhibition by combined application of indomethacin and nordihydroguaiaretic acid. *Cancer Detect Prev* 2005; **29**:276–285.
40 Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006; **58**:621–681.
41 Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc* 2006; **1**:2315–2319.
42 Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Prot* 2007; **2**:329–333.
43 Cortez MA, Nicoloso MS, Shimizu M, Rossi S, Gopisetty G, Molina JR, *et al*. miR-29b and miR-125a regulate podoplanin and suppress invasion in glioblastoma. *Genes Chromosomes Cancer* 2010; **49**:981–990.
44 Horiuchi K, Morioka H, Nishimoto K, Suzuki Y, Susa M, Nakayama R, *et al*. Growth suppression and apoptosis induction in synovial sarcoma cell lines by a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ). *Cancer Lett* 2008; **272**:336–344.
45 Horiguchi Y, Kuroda K, Nakashima J, Murai M, Umezawa K. Antitumor effect of a novel nuclear factor-kappa b activation inhibitor in bladder cancer cells. *Expert Rev Anticancer Ther* 2003; **3**:793–798.
46 Hafeez BB, Ahmed S, Wang N, Gupta S, Zhang A, Haqqi TM. Green tea polyphenols-induced apoptosis in human osteosarcoma SAOS-2 cells involves a caspase-dependent mechanism with downregulation of nuclear factor-κB. *Toxicol Appl Pharmacol* 2006; **216**:11–19.
47 Tanaka H, Yoshida K, Okamura H, Morimoto H, Nagata T, Haneji T. Calyculin A induces apoptosis and stimulates phosphorylation of p65NF-kappaB in human osteoblastic osteosarcoma MG63 cells. *Int J Oncol* 2007; **31**:389–396.
48 Karin M. Nuclear factor-B in cancer development and progression. *Nature* 2006; **441**:431–436.
49 Nakanishi C, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005; **5**:297–309.
50 Clover J, Gowen M. Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype? *Bone* 1994; **15**:585–591.
51 Poma P, Notarbartolo M, Labbozzetta M, Sanguedolce R, Alaimo A, Carina V, *et al*. Antitumor effects of the novel NF-kappaB inhibitor dehydroxymethyl-epoxyquinomicin on human hepatic cancer cells: analysis of synergy with cisplatin and of possible correlation with inhibition of pro-survival genes and IL-6 production. *Int J Oncol* 2006; **28**:923–930.
52 Grivennikov SI, Karin M. Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage. *Ann Rheum Dis* 2011; **70 (Suppl 1):**i104–i108.
53 Jazirehi A, Vega M, Bonavida B. Development of rituximab-resistant lymphoma clones with altered cell signaling and cross-resistance to chemotherapy. *Cancer Res* 2007; **67**:1270–1281.
54 Jazirehi A, Huerta-Yepez S, Cheng G, Bonavida B. Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor-kB signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis. *Cancer Res* 2005; **65**:264–276.
55 Vega MI, Huerta-Yepez S, Martinez-Paniagua M, Martinez-Miguel B, Hernandez-Pando R, González-Bonilla CR, *et al*. Rituximab-mediated cell signaling and chemo/immuno-sensitization of drug-resistant B-NHL is independent of its Fc functions. *Clin Cancer Res* 2009; **15**:6582–6594.
56 Ng CP, Bonavida BA. New challenge for successful immunotherapy by tumors that are resistant to apoptosis: two complementary signals to overcome cross-resistance. *Adv Cancer Res* 2002; **85**:145–174.
57 Ohsugi T, Kumasaka T, Ishida A, Ishida T, Horie R, Watanabe T, *et al*. In vitro and in vivo antitumor activity of the NF-kappaB inhibitor DHMEQ in the human T-cell leukemia virus type I-infected cell line, HUT-102. *Leuk Res* 2006; **30**:90–97.