Efficacy of a nitrogen-containing bisphosphonate, minodronate, in conjunction with a p38 mitogen activated protein kinase inhibitor or doxorubicin against malignant bone tumor cells

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Abstract

Purpose We recently reported the sarcoma-selective antitumor effects of a newly developed nitrogen-containing bisphosphonate, minodronate (MIN) on malignant bone tumors. The aim of this study was to develop efficient combination MIN therapy in malignant bone tumors.

Methods We examined downstream molecular events of MIN in osteosarcoma and Ewing's sarcoma cells to search for a partner to combine with MIN. Furthermore, we evaluated the combined effects of MIN and clinically available Doxorubicin (DOX).

Results We found that MIN inhibited Rap 1A prenylation, and extracellular signal-regulated kinase (ERK) or Akt phosphorylation in osteosarcoma (Saos-2) and Ewing's sarcoma (SK-ES-1) cells. Interestingly, MIN activated p38 mitogen activated protein kinase (MAPK) only in SK-ES-1 cells and a p38 MAPK inhibitor augmented MIN-induced growth inhibition in SK-ES-1 cells. Doxorubicin (DOX) exerted synergistic effects on Saos-2 and SK-ES-1 cell lines. Daily injection of MIN enhanced the growth inhibition of SK-ES-1 xenograft sarcoma treated by DOX in nude mice.

Conclusions These findings suggest that the inhibition of the p38 MAPK pathway may be attractive in overcoming cellular resistance against MIN. In the light of clinical settings, MIN may have a beneficial adjuvant role in the DOX treatment.

Keywords Bisphosphonates – Osteosarcoma - Ewing's sarcoma – Doxorubicin - p38 mitogen activated protein kinase

Abbreviations MIN Minodronate - ERK Extracellular signal-regulated kinase – MAPK Mitogen activated protein kinase - DOX Doxorubicin - FBS Fetal bovine serum - PBS Phosphate buffered saline

Introduction

Osteosarcoma and Ewing's sarcoma are the two most common types of primary bone malignancy in childhood and adolescence. Despite significant improvement in patient survival rates by intensifying chemotherapy protocols, some groups of patients with unresectable primary tumors, like intense pelvic lesion, and those with clinically evident metastases still have a poor prognosis even in chemotherapy-responsive osteosarcoma and Ewing's sarcoma [1,2]. Thus, the development of more effective therapeutic approaches for these refractory subgroups is warranted.

Bisphosphonates, stable analogues of pyrophosphonate, are potent inhibitors of osteoclast-mediated bone resorption and integral in the treatment of bone diseases such as osteoporosis and bone metastases. Moreover, increasing preclinical evidence that bisphosphonates have direct antitumor effects on a variety of human cancer cells has been reported [3,4]. We recently reported the powerful antitumor effects of a newly developed nitrogen-containing bisphosphonate, minodronate (MIN) on osteosarcoma, chondrosarcoma, and Ewing's sarcoma cells, whereas MIN affected the viability of normal bone marrow stromal cells much less than sarcoma cells. These findings suggest that MIN may have a beneficial adjuvant role in the treatment of patients with various malignant bone tumors. However, MIN alone could not eradicate sarcomas in xenografted mice [5].

Nitrogen-containing bisphosphonates are generally considered to exert their biological effects on osteoclasts and tumor cells by inhibiting the mevalonate pathway, preventing

post-translational events of prenylation of small GTP-binding proteins, such as Ras, Rho, and Rap 1A, which play a role in the malignant transformation of cells. The aim of this study was to examine downstream molecular events of MIN in osteosarcoma (Saos-2) and Ewing's sarcoma (SK-ES-1) cells to search for a partner to combine with MIN. In the light of clinical settings, we also determined whether a conventional chemotherapeutic drug, doxorubicin (DOX), can enhance those antiproliferative effects of MIN in osteosarcoma and Ewing's sarcoma cells, and xenografts in nude mice.

Materials and methods

Cell culture and reagents

A human osteosarcoma (Saos-2) cell line and a human Ewing's sarcoma (SK-ES-1) cell line were purchased from the American Type Culture Collection (Manassas, VA). Saos-2 cells and SK-ES-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and McCoy's 5A medium with 15% FBS, respectively. MIN (1-hydroxy-2-(imidazo[1,2-a]pyridin-3-yl)ethylidene bisphosphonic acid monohydrate) and DOX were kindly supplied by Yamanouchi Pharmaceuticals (Ibaragi, Japan) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively. SB203580, a specific inhibitor of the p38 mitogen activated protein kinase (MAPK), was purchased from Calbiochem (San Diego, CA).

Immunoblotting

Cells were incubated for 24 h with 50 μM MIN or with phosphate buffered saline (PBS) as a control. Cells were lysed in M-PER^{TM} Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Whole cell lysates were separated by SDS-polyacrylamide gel

electrophoresis under reducing conditions. Proteins were electrophoretically transferred to Hybond-P PVDF membranes (Amersham Biosciences, NJ) and immunoblotted with anti-unprenylated Rap 1A antibody (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Actin antibody (diluted 1:200; Santa Cruz Biotechnology), anti-phospho Akt antibody, anti-phospho extracellular signal-regulated kinase (ERK) 1/2 antibody, anti-phospho p38 MAPK antibody, anti-Akt antibody, anti-ERK1/2 antibody, or anti-p38 MAPK antibody (diluted 1:1,000, Cell Signaling Technology, Beverly, MA). Bound antibodies were visualized using the enhanced chemiluminescence system (ECL Plus Western Blotting Detection System, Amersham Biosciences).

Cytotoxicity assay

Cells were grown in the appropriate medium supplemented with 10% FBS in 96–well culture plates at a density of 5 x 10^3 cells/well and allowed to adhere overnight. Cells were then treated for 48 h with MIN (1, 5, and 50 μM), DOX (0.1, 1, 10, 100, and 1000 nM), SB203580 (1 μM), or in combination. The viable cell amount was measured by Cell Counting Kit-8 (Dojin, Kumamoto, Japan), according to the instructions. In brief, a 10% working solution including 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8) was added into each well, and cultures were incubated for 4 h at 37°C. The absorbance was measured at 450 nm (reference wavelength 690 nm) using a microplate reader (SPECTRAmax190, Molecular Devices, Sunnyvale, CA). Cell viability was expressed as a percentage of the absorbance of treated cells vs. untreated cells.

In vivo effects of MIN and DOX

Xenografts of human SK-ES-1 cells were initiated by subcutaneous injections of 1 X 10^7 cells in 100 μl of McCoy's 5A medium into the right flanks of 6 athymic nude mice at the age of 6 weeks (CLEA, Tokyo, Japan). One week after inoculation, the mice were pair-matched into four experimental groups (6 mice/group) as follows; physiological saline-treated control; MIN alone; DOX alone; and MIN + DOX. The MIN alone and MIN + DOX groups received daily intraperitoneal injections of MIN (100 μg/kg). The DOX alone and MIN + DOX groups received a single intravenous administration of DOX (5 mg/kg), which was repeated once per week for a total of three treatments (15 mg/kg). The smallest and largest diameters of tumors, and the body weights were measured once a week. Tumor volumes were calculated using the following formula: volume (mm^3) = (smallest diameter)^2 X (largest diameter)/2. All animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee and the protocol was approved by the Ethics Committee for Experimental Animals of Hiroshima University.

Statistical analysis

Statistical significance was determined by Student's *t*-test, using SPSS (Chicago, IL), and one-way ANOVA and Fisher's PLSD method, using Statcel (OMS Ltd., Saitama, Japan); $p < 0.05$ was considered to be significant. The combined effects of MIN and DOX were determined by isobolographic analysis, as reported by Berenbaum [6,7]. The nature of the effect of a particular dose combination was analyzed by an isobologram, as follows: the point representing that combination would lay on, below, and above the straight line

joining the doses of the two drugs that, when given alone, produce the same effect as that combination, representing additive, synergistic, and antagonistic effects, respectively.

Results

Molecular effects of MIN on the signaling cascade from small GTP-binding proteins

We examined responses of downstream signaling molecules from small GTP-binding proteins to the MIN treatment. MIN inhibited Rap 1A prenylation in Saos-2 cells and SK-ES-1 cells. MIN induced marked decreases in the levels of the Akt phosphorylation in SK-ES-1 cells and slight decreases in the levels of the phosphorylated forms of ERK in Saos-2 cells. In contrast, MIN treatment resulted in increased phosphorylation of p38 MAPK in SK-ES-1 cells. In all cases, equal protein loading was confirmed by demonstrating equal levels of Actin or phosphorylation state-independent forms of the individual molecules (Fig. 1).

Combination therapy of MIN and a p38 MAPK inhibitor

Immunoblotting results prompted us to further investigate the combined therapeutic effects of MIN and p38 MAPK inhibition. MIN inhibited the viability of Saos-2 cells and SK-ES-1 cells in a concentration-dependent manner, whereas a specific inhibitor of the p38 MAPK, SB203580 alone (1 μM) did not have any effect on Saos-2 and SK-ES-1 cell growth. Simultaneous addition of SB203580 with MIN significantly augmented growth inhibition in SK-ES-1 cells, but not in Saos-2 cells (p <0.05, Fig. 2).

In vitro combination therapy of MIN and DOX

DOX is commonly used in conventional chemotherapy regimens to manage

osteosarcoma and Ewing's sarcoma. Several clinical pharmacokinetic studies have shown that following an intravenous bolus administration of DOX, plasma levels of 1 μM are attainable [8,9]. Thus, we used clinically relevant concentrations of <1 μM DOX. Exposure of Saos-2 cells or SK-ES-1 cells to DOX alone was confirmed to cause a dose-dependent inhibition of cell growth (Fig. 3). Isobolographic analysis showed that DOX combined with MIN exerted synergistic combination effects on both Saos-2 cells and SK-ES-1 cells (Fig. 4).

In vivo combination therapy of MIN and DOX

Instead of orthotopical implantation of SK-ES-1 cells into the tibia of nude mice, we used subcutaneous implantation of SK-ES-1 cells in nude mice, because there was no evidence of intraosseous tumor formation at 6 weeks after intratibial implantation. Also, Saos-2 cells were not tumorigenic in nude mice.

Fig. 5 summarized tumor growth rates of mice treated with control vehicle, MIN, DOX, or MIN + DOX. SK-ES-1 xenograft sarcoma cells in the control group grew rapidly in nude mice. MIN + DOX was clearly more effective in reducing tumor volume as compared with treatment with MIN alone, DOX alone, or control. Four weeks after tumor inoculation, the mean tumor volume was 515 mm^3 in the MIN + DOX group, which was significantly smaller than that of the control group (1261 mm^3), the MIN group (1008 mm^3), and the DOX group (914 mm^3). ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively; one-way ANOVA and Fisher's PLSD method). All mice survived without significant differences of body weights among all groups throughout the treatment period.

Discussion

In general, nitrogen-containing bisphosphonates, including MIN, are considered to inhibit the mevalonate pathway, which prevent prenylation of small GTP-binding proteins such as Ras, Rho, and Rap 1A and suppress activation of intracellular signaling proteins such as ERK1/2 and Akt, ultimately leading to apoptosis in tumor cells as well as in osteoclasts. However, there are conflicting results about the details of the molecular mechanisms of the antiproliferative action of bisphosphonate and the bisphosphonate-inducing apoptosis [10-13]. Our previous report demonstrated that MIN treatment resulted in S phase arrest and apoptosis in Saos-2 cells but induced cell death without the induction of apoptosis in SK-ES-1 cells [5]. Likewise, the current study showed that MIN inhibited Rap 1A prenylation in Saos-2 cells as well as in SK-ES-1 cells, although MIN suppressed ERK and Akt phosphorylation in Saos-2 and SK-ES-1 cells, respectively, suggesting that MIN induce different molecular events in the Ras signaling pathway among cell lines.

More interestingly, MIN activated p38 MAPK in SK-ES-1 cells and the specific inhibitor of the p38 MAPK (SB203580) augmented MIN-induced growth inhibition in SK-ES-1 cells. The p38 MAPK pathway is activated in response to cellular stress, such as UV light, ionizing radiation, and cytotoxic drugs [14-16]. p38 MAPK-mediated drug-resistance has been described in mouse leukemic cells in response to vincristine [17]. Merrell et al. [18] reported that bisphosphonate activate the p38 MAPK in some breast cancer cell lines which are resistant to the growth inhibitory effects of bisphosphonates, whereas no such p38 activation nor augmentation of bisphosphonate-induced growth inhibition were

detected in sensitive cell lines. Our findings suggested that since addition of SB203580 alone did not affect viability in either cell line, and simultaneous addition of SB203580 to MIN did not augment growth inhibition in Saos-2 cells where MIN did not induce phosphorylation of p38 MAPK, activation of p38 MAPK by MIN may be protecting SK-ES-1 cells from further drug-induced damage. In such cases, a selective p38 MAPK inhibitor may be a promising agent in combination with MIN in the near future. A huge effort was initiated by many pharmaceutical companies to develop p38 MAPK inhibitors such as AMG-548 (Amgen Inc.) and BIRB-796 (Boehringer Ingelheim Pharmaceuticals Inc.) as potential treatments for inflammatory diseases and have advanced to clinical trials [19].

Our present study showed that the combination of DOX with MIN enhanced the inhibitory effects of the clinically relevant concentrations of DOX on the proliferation of Saos-2 cells and SK-ES-1 cells. DOX is a complex drug in terms of its cytotoxic mechanisms of action, although it is known to be cell cycle-specific in a variety of cell lines. The mechanism by which bisphosphonates enhance the effects of anti-cancer agents is not fully understood, however, some authors speculated the possibility of bisphosphonates as a potential cell cycle chemosensitizer [20,21]. MIN concentrations used in this experiment ranged from 1 μM to 50 μM. Based on the pharmacokinetics of clinical trials with ZOL for cancer patients with bone metastases, a potentially achievable serum concentration of bisphosphonate could be 1 - 2 μM [22]. According to the data of a rat experiment, the effective local concentrations of bisphosphonates at sites of active bone resorption could reach up to 1 mM, due to their great avidity for bone [23]. Our

study also demonstrated that daily injection of MIN enhanced the DOX-induced growth inhibition of subcutaneous xenograft sarcoma in nude mice. Although there is no convincing evidence that MIN can have a direct effect on tumor cells themselves within the bone microenvironment, malignant bone tumors may be a more favorable target of MIN than subcutaneous sarcoma and our findings may have relevance for clinical settings. Our findings should prompt further investigations using orthotopic xenograft models to confirm our findings within the bone microenvironment.

In conclusion, we demonstrated that MIN caused different molecular events in the signaling pathway from small GTP-binding proteins between osteosarcoma (Saos-2) and Ewing's sarcoma (SK-ES-1) cells. The inhibition of the p38 MAPK pathway may be attractive in overcoming cellular resistance against MIN in some malignant tumor cells. The combination of MIN with the standard chemotherapeutic drug DOX may be useful for patients with malignant bone tumors.

Acknowledgements

This work was supported in part by the Grants-in-Aids for Scientific Research (C) 1659149100 from the Ministry of Education, Science, Sports, Culture, and Technology of Japan.

No benefits in any form have been received from a commercial party related directly or indirectly to the subject of this article.

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Figure Legends

Fig. 1 Minodronate (MIN) down-regulated the signaling cascade from small GTP-binding proteins, except for increased phosphorylation of p38 MAPK (p-p38) in SK-ES-1 cells. Cells were treated for 24 h with 50 μM MIN and the effects of MIN on signaling molecules were analyzed by immunoblotting. Equal protein loading was confirmed by demonstrating equal levels of unmodified molecules. Each experiment was performed in triplicate with equivalent results. u-Rap1A, unprenylated form of Rap1A.

Fig. 2 Inhibition of p38 MAPK activity enhanced the cytotoxicity of minodronate (MIN) in SK-ES-1 (b), but not in Saos-2 cells (a). Cells were treated for 48 h with the indicated concentrations of MIN with or without a p38 MAPK inhibitor (1 μM SB203580). The viable cell amount was assessed by the colorimetric WST-8 method. Data show the mean ± SD of four independent experiments. $*p < 0.05$ vs. control group (untreated cells) as determined by Student's *t*-test.

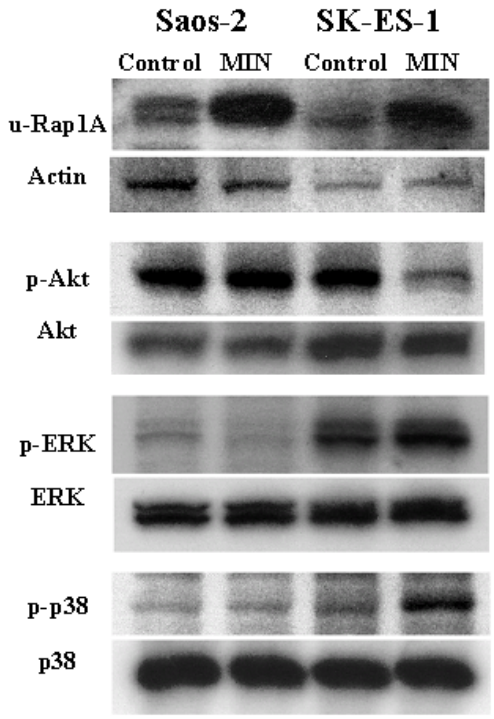
Fig. 3 Combination therapy of minodronate (MIN) and doxorubicin (DOX) against Saos-2 (a) and SK-ES-1 cells (b). Cells were treated for 48 h with the indicated dose of DOX in combination with the indicated dose of MIN. Cell growth was determined by colorimetric WST-8 assay. Data shown are the mean ± SD of four independent experiments.

Fig. 4 Synergistic cytotoxicity of minodronate combined with doxorubicin against Saos-2 (a) and SK-ES-1 (b). The data shown in Fig. 3 were assessed by isobolographic analysis.

The point representing that combination would lay on, below, and above the straight line representing additive, synergistic, and antagonistic effects, respectively.

Fig. 5 Effects of minodronate (MIN) and doxorubicin (DOX), alone and in combination on xenograft growth of human SK-ES-1 sarcoma cells in nude mice. One week after tumor inoculation, the MIN alone and MIN + DOX groups received daily intraperitoneal injections of MIN (100 μg/kg). The DOX alone and MIN + DOX received a single intravenous administration of DOX (5 mg/kg), which was repeated once per week. The control mice were injected with the same volume of physiological saline. Data are mean ± SD obtained from 6 athymic mice. * $p < 0.05$; ** $p < 0.01$ as determined by one-way ANOVA and Fisher's PLSD method.

Fig. 1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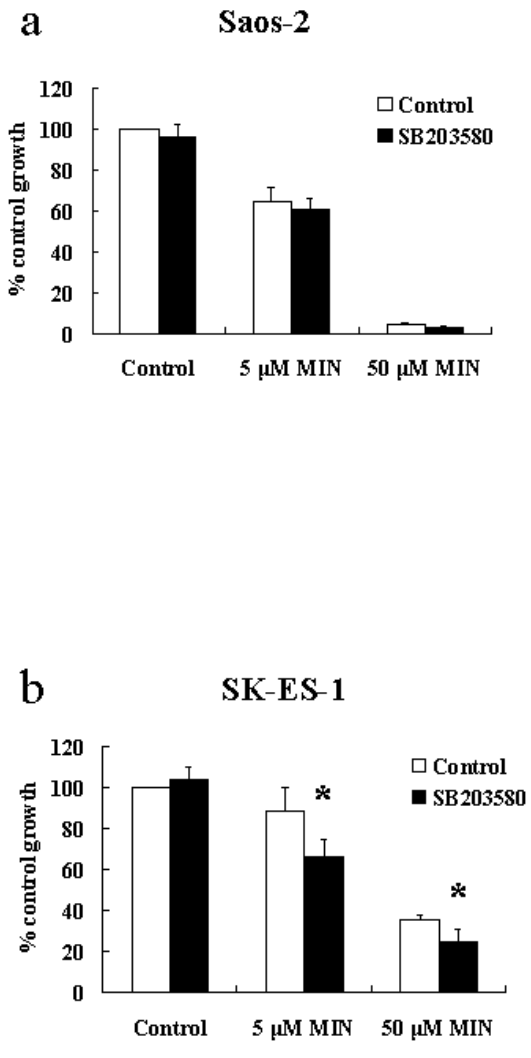
p38

Fig. 2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Saos-2
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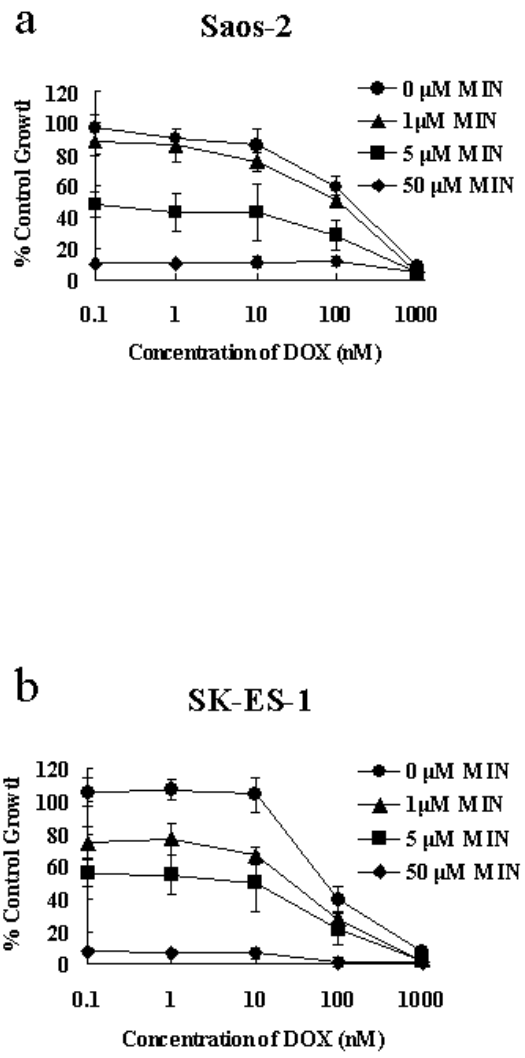
Fig. 3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Concentration of DOX (nM)
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1μM MIN
5 μM MIN
50 μM MIN
b
SK-ES-1
% Control Growth
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Concentration of DOX (nM)
0 μM MIN
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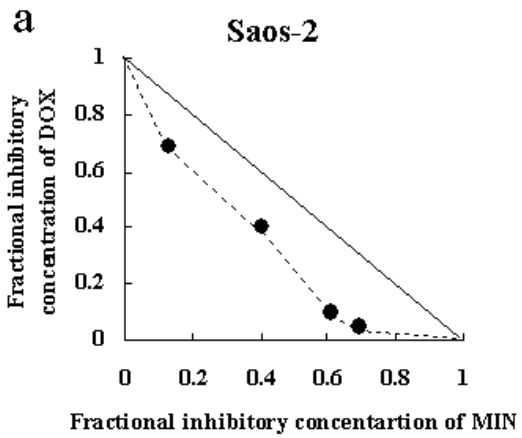
5 μM MIN
50 μM MIN

Fig. 4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Fractional inhibitory concentration of DOX
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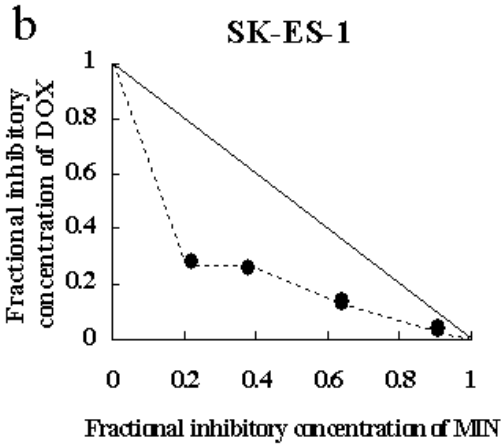
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SK-ES-1
Fractional inhibitory concentration of DOX
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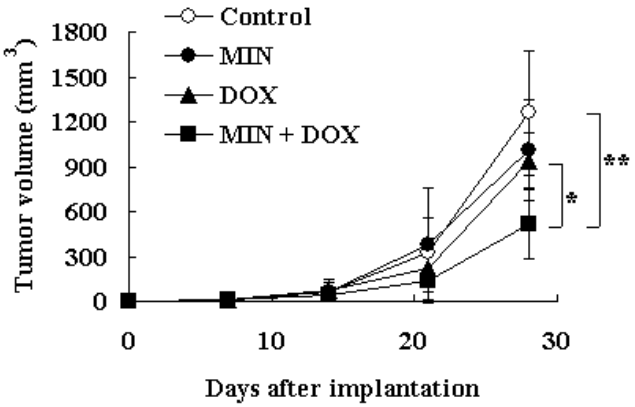
1
Fractional inhibitory concentration of MIN

Fig. 5


Control
MIN
DOX
MIN + DOX
Tumor volume (mm3)
1800
1500
1200
900
600
300
0
0
10
20
30
Days after implantation
*
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