BH3 Mimetics Inhibit Growth of Chondrosarcoma – A Novel Targeted-therapy for Candidate Models

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Abstract. *Background: Chondrosarcoma is refractory to conventional chemotherapy. BH-3 mimetics ABT-737 and ABT-263 are synthetic small-molecule inhibitors of anti-apoptotic proteins B-cell lymphoma-2 (Bcl2) and Bcl-xL, which play a critical role in survival of chondrosarcoma cells. Materials and Methods: Chondrosarcoma cell lines SW-1353 and CS-1 were used as the disease model. We used immunoblotting to assess the expression of target molecules Bcl2 and Bcl-xL, and the apoptotic inducers Bcl2-associated X (Bax) and Bcl2-antagonist/killer (Bak). In vitro growth inhibition by BH-3 mimetics was confirmed by photomicroscopic cell counting and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Apoptotic induction was confirmed by Enzyme-Linked ImmunoSorbent Assay (ELISA). In vivo growth inhibition was assessed in a non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse model. Results: Expression of the target and effector molecules was confirmed in chondrosarcoma cell lines. BH3 mimetics significantly inhibited cell growth and induced apoptosis in vitro. Administration of ABT-263 inhibited chondrosarcoma growth and improved survival in a mouse model. Conclusion: BH3 mimetics represent a novel treatment modality for chondrosarcoma.*

Chondrosarcoma is the second most common primary malignant bone tumor characterized by cartilage formation. It is refractory to conventional chemotherapy and radiotherapy, and the only way to control disease progression is through surgical resection with adequate margins (1). Cases with systemic dissemination or with tumors located adjacent to unresectable vital organs are clinically-challenging and there is a great need for novel therapeutic modalities to control disease progression.

Apoptosis is a mechanism of controlled cell deletion, which plays a complementary but opposite role to mitosis in the regulation of animal cell populations (2). In several cell types, including cancer, apoptosis is regulated by interactions between members of the B-cell lymphoma-2 (Bcl2) protein family, which represents α-helical proteins that carry conserved Bcl2 homology (BH) domains and can function as pro-survival or pro-apoptotic molecules (3). Pro-survival molecules Bcl2, Bcl-xL, and myeloid cell leukemia 1 (Mcl1) regulate apoptosis in cancer cells (4). The pro-apoptotic members of the family can be sub-divided into two functionally and structurally distinct classes; Bcl2-associated X (Bax)/Bcl2-antagonist/killer (Bak) and the BH3-only proteins. The latter, including Bcl2 interacting mediator of cell death (Bim), p53 up-regulated modulator of apoptosis (Puma), BH3 interacting domain death agonist (Bid), Bcl2-associated agonist of cell death (Bad), Bcl2-interacting killer (Bik), Bcl2 modifying factor (Bmf), harakiri (Hrk), and Noxa, share only the BH3 region of homology and serve as upstream sentinels, becoming activated in response to various forms of cellular stress, whereas the former contain several BH domains (BH1, BH2 and BH3) and are required downstream of BH3-only proteins to induce apoptosis (3).

The Bcl2 family of proteins function in the establishment and progression of chondrosarcoma. In contrast to osteochondroma, a benign counterpart of chondrosarcoma, Bcl2 is overexpressed and the expression level increases with progression of histological grade in chondrosarcoma (5, 6). Altered expression of Bcl-xL and Mcl1 is closely associated with growth inhibition of chondrosarcoma cell lines *via* down-regulation of phospho-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) and phospho-extracellular signal-regulated kinase (ERK) (7). Thus, the Bcl2 family might be promising therapeutic targets in chondrosarcoma.

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Key Words: Chondrosarcoma, apoptosis, BH3 mimetics, Bcl2.

ABT-737 is a synthetic small-molecule inhibitor produced by Nuclear Magnetic Resonance-guided, structure-based drug design; it binds to Bcl2, Bcl-xL, and Bcl-W with high affinity but binds weakly to other anti-apoptotic Bcl2 family members, including Mcl1 and Bcl-2 fetal liver (Bfl)1 (8). This small molecule disrupts Bcl2–Bax heterodimerization and induces a conformational change in Bax, thereby inducing apoptosis and enhancing chemotherapeutic efficacy in many kinds of cancer cells (9, 10). ABT-263, a second-generation orally-bioavailable BH3 mimetic, is similar to ABT-737 in its capacity to inhibit cancer progression (4). In a previous study, the possibility for enhancement of conventional chemotherapy effect by ABT-737 on chondrosarcoma was shown (11) but ABT-263 has not been explored in this context. In addition, no *in vivo* model of chondrosarcoma as a target of BH3 mimetics has been studied. In the present study, we demonstrated the *in vitro* and *in vivo* effects of ABT-737 and ABT-263 on chondrosarcoma cell growth in order to develop a novel treatment model of adjuvant therapy targeting the Bcl2 family.

Materials and Methods

Cell lines and reagents. The chondrosarcoma cell line CS-1 was derived from a 62-year-old male with metastatic chondrosarcoma (12). The patient had no previous exposure to radiation or chemotherapy. mRNA for type II collagen and annual cytogenetic analysis was performed. The cells were cultured in RPMI-1640 with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2. The human chondrosarcoma cell line SW 1353 was obtained from the American type culture collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS in a humidified atmosphere of 5% CO_2. ABT-263 and ABT-737 were purchased from SelleckBio (Houston, TX, USA).

Growth curve. Cells (1×10^4/well) were seeded into 6-well plates. After overnight incubation, the BH-3 mimetics (0-30 μM) were added and cell proliferation was evaluated on culture days 1-4 by photomicroscopic cell counting in a low-power field.

Cell proliferation. Cell proliferation was monitored by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay as described in a previous study, with modifications (13). Cells were seeded in 96-well plates at 3,000 cells/well and treated with test reagents after 24-h incubation. After 72 h, the viable cells were detected by spectrophotometric CellTiter 96 aqueous cell proliferation assay (Promega, Madison, WI, USA).

Immunoblotting. Cell lysates (10 μg protein/sample) were resolved by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed with an affinity-purified monoclonal antibody against Bcl2 (sc-7382) and Bcl-xL (sc-8392; Santa Cruz Biotechnology, Santa Cruz, CA, USA); Mcl-1 (#4572), Bax (#5023), Bak (#3814), Bad (#9239), Bim (#2933), and Bid (#2002; Cell Signaling Technology, Danvers, MA, USA), and beta-actin (M177-3; Medical & Biological Laboratories, Nagoya, Japan). The blot was incubated with horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) and HRP-linked secondary antibody (Cell Signaling Technology), and fluorescence detection was performed using LumiGLO peroxidase chemiluminescent substrate kit (KPL, Gaithersburg, MD, USA).

Detection of apoptosis by Enzyme-Linked ImmunoSorbent Assay (ELISA). ELISA was used for apoptosis detection as described previously (13). In brief, cells were seeded in 96-well plates at 3,000 cells/well and incubated for 24 h. After washing twice with phosphate-buffered saline (PBS), medium containing test agents was added. After 48 h incubation, apoptosis was detected by ApoStrand™ ELISA Apoptosis Detection (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA).

In vivo anti-chondrosarcoma activity of BH3 mimetics. Animal studies were performed according to the guidelines of the Institutional Review Board for Animal Studies of Kyorin University. To generate a murine tumor model, 5×10^6 SW1353 cells in 0.2 ml 50% Matrigel (BD Biosciences, Bedford, MA, USA) were injected subcutaneously into the right limb of 10 4-week-old male non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (The Jackson Laboratory, Bar Harbor, ME, USA). The mice were ear-tagged and individually monitored as described elsewhere (4,14), with modification. One week after injection, the subcutaneous tumor size had reached a diameter of approximately 4 mm, and five mice were treated with two 5-day courses of intraperitoneal (*i.p.*) injections of 50 mg/kg ABT-263 (*i.e.* injections on days 8-12 and 18-22). The 5-day interval was established to permit platelet recovery (15, 16). The five remaining mice received 200 μl/day PBS *via i.p.* injection. The tumor mass was measured weekly, with volumes calculated as follows: length × width^2 × π/6 (14). Mice were sacrificed when they became moribund, unable to obtain food or water, lost >20% of their body weight, or tumor volume reached more than 4000 mm3.

Statistical analysis. All data are expressed as the mean±SD. Inter-group differences were analyzed by Mann–Whitney *U*-test. Survival curves were drawn using the Kaplan–Meier method and compared with the log-rank test. A value of $p<0.05$ was considered statistically significant.

Results

To understand the potential therapeutic efficacy of BH3 mimetics in chondrosarcoma, we studied the protein expression of the Bcl2 family, including anti-apoptotic BH3 targets Bcl2 and Bcl-xL and pro-apoptotic BH3 effectors Bax and Bak (9, 17). As shown in Figure 1, expression of Bcl2, Bcl-xL, Mcl1, Bax, Bak, Bim, and Bid was confirmed (Figure 1), but expression of Bad was not detected (data not shown). These data suggest that the anti-apoptotic proteins Bcl2 and Bcl-xL may play a role in the survival of these chondrosarcoma cell lines.

Based on the findings regarding the balanced expression of Bcl2 proteins and apoptosis regulators, we speculated that

BH3 mimetics might regulate chondrosarcoma cell growth, and the effect of BH3 mimetics on *in vitro* cell growth was analyzed. Indeed in both photomicroscopic cell counting analysis and MTS assays, treatment with different concentrations of ABT-263 and ABT-737 inhibited chondrosarcoma cell growth in a dose- and a time-dependent manner (Figure 2, and Figure 3 A-D).

The effects of BH3 mimetics were reported to be through induction of apoptosis (9, 10). Next we confirmed such apoptosis induction by the BH3 mimetics in the cell lines. As expected, enhancement of apoptosis induction by the treatment with BH3 mimetics was confirmed in ELISA for apoptosis detection (Figure 3E-H).

Finally, to test chondrosarcoma sensitivity to BH3 mimetics *in vivo*, tumor growth of SW1353 chondrosarcoma xenografts in NOD/SCID mice treated with ABT-263 was analyzed. Beginning at day 7, mice were treated with the indicated volume of ABT-263 (4,15). ABT-263 treatment significantly inhibited chondrosarcoma growth (p=0.02 on days 35 and 42, Figure 4A-C) and significantly extended survival (p=0.002, Figure 4D). There was no significant body weight loss neither in the control nor in the ABT-263-treated group (data not shown).

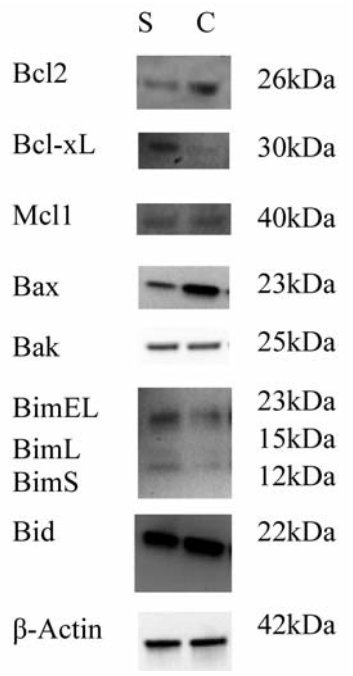

Figure 1. *Basal protein expression of the B-cell lymphoma (Bcl2) family in CS-1 (C) and SW1353 (S) cells by western blotting. Mcl1; Myeloid cell leukemia 1, Bax; Bcl2-associated X, Bak; Bcl2-antagonist/killer, Bim; Bcl2 interacting mediator of cell death, Bid; BH3 interacting domain death agonist.*

Discussion

Bcl2 protein expression is associated with tumor progression and chondrosarcoma tumorigenesis. Bcl2 and parathyroid hormone-related protein are up-regulated during malignant transformation of osteochondroma (the most frequent primary benign bone tumor and counterpart of chondrosarcoma) into low-grade chondrosarcoma (peripheral chondrosarcoma) (5, 18, 19). Bcl2 is expressed in peripheral and high-grade central chondrosarcoma; it inhibits apoptosis and the downstream effector of the Indian hedgehog pathway (5, 20). Universal expression of Bcl2 including Bcl2 (7, 21, 22), Bcl-xL (7, 21, 23), Mcl1 (7). and Bax (7, 22, 23) in chondrosarcoma was also confirmed. Extracellular stimulation of tumor growth inhibition induces apoptosis *via* up-regulation of Bax and down-regulation of Bcl2 in chondrosarcoma cell lines (7, 22, 23). Resistance to conventional chemotherapeutic agents has been reported in response to the anti-apoptotic activity of Bcl2 (11). Thus, the Bcl2 family proteins are promising therapeutic targets in chondrosarcoma.

Bcl2 and Bcl-xL form heterodimers with a number of pro-apoptotic proteins such as Bak, Bad, and Bax; they are thus anti-apoptotic (3, 24). Inhibitory compounds dock with the BH3 domain of Bcl2 or Bcl-xL, thus re-activating the pro-apoptotic proteins Bax/Bak; such compounds have been the subject of intense study (9). ABT-737 is a synthetic small-molecule inhibitor with high affinity for Bcl2 and Bcl-xL, produced by nuclear magnetic resonance-based screening, parallel synthesis, and structure-based design (8). ABT-737 binds with high affinity to Bcl-xL, Bcl2, and Bcl-W ($K_i \leq 1$nM), but not to the less homologous proteins Bcl-B, Mcl1, and A1; it antagonizes Bcl2 protection at concentrations greater than 10 nM. Nanomolar activity was retained in the presence of 10% human serum (8). Presence of Bax or Bak proved sufficient to mediate the effects of ABT-737. Indeed anti-tumor effects by this compound *in vitro* and *in vivo* have been reported in acute myeloid leukemia (9), chronic myeloid leukemia (15), lung cancer (25), breast cancer (26), renal cell carcinoma (27), and head and neck cancer (26). However, for chondrosarcoma, only one *in vitro* report has been published so far (11).

ABT-263 is a second-generation orally-bioavailable BH3 mimetic with selective *in vitro* cytotoxicity in Bcl2/Bcl-xL-dependent cells (4). It induces Bax translocation and cytochrome c release, thus inducing apoptosis. Oral administration of ABT-263 alone induces complete tumor regressions in xenograft models of small-cell lung cancer and acute lymphoblastic leukemia (4). This agent is currently undergoing phase I study (28). No study has yet been published regarding the effects of this compound on chondrosarcoma.

These agents may also enhance the effect of conventional chemotherapeutic agents. Van Oosterwijk *et al*. reported the *in vitro* effect of ABT-737 in chondrosarcoma and concluded

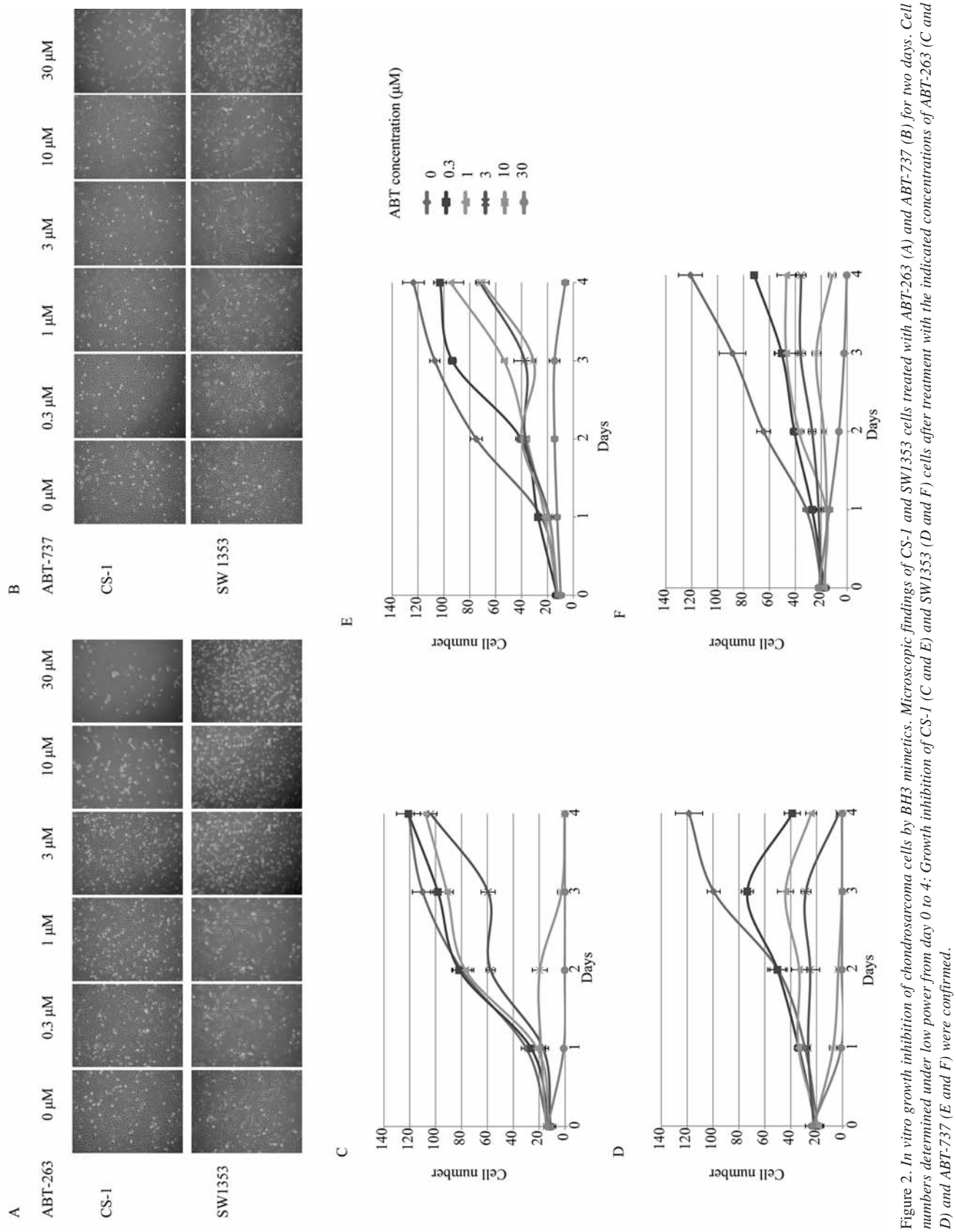

Figure 2. *In vitro growth inhibition of chondrosarcoma cells by BH3 mimetics. Microscopic findings of CS-1 and SW1353 cells treated with ABT-263 (A) and ABT-737 (B) for two days. Cell numbers determined under low power from day 0 to 4: Growth inhibition of CS-1 (C and E) and SW1353 (D and F) cells after treatment with the indicated concentrations of ABT-263 (C and D) and ABT-737 (E and F) were confirmed.*

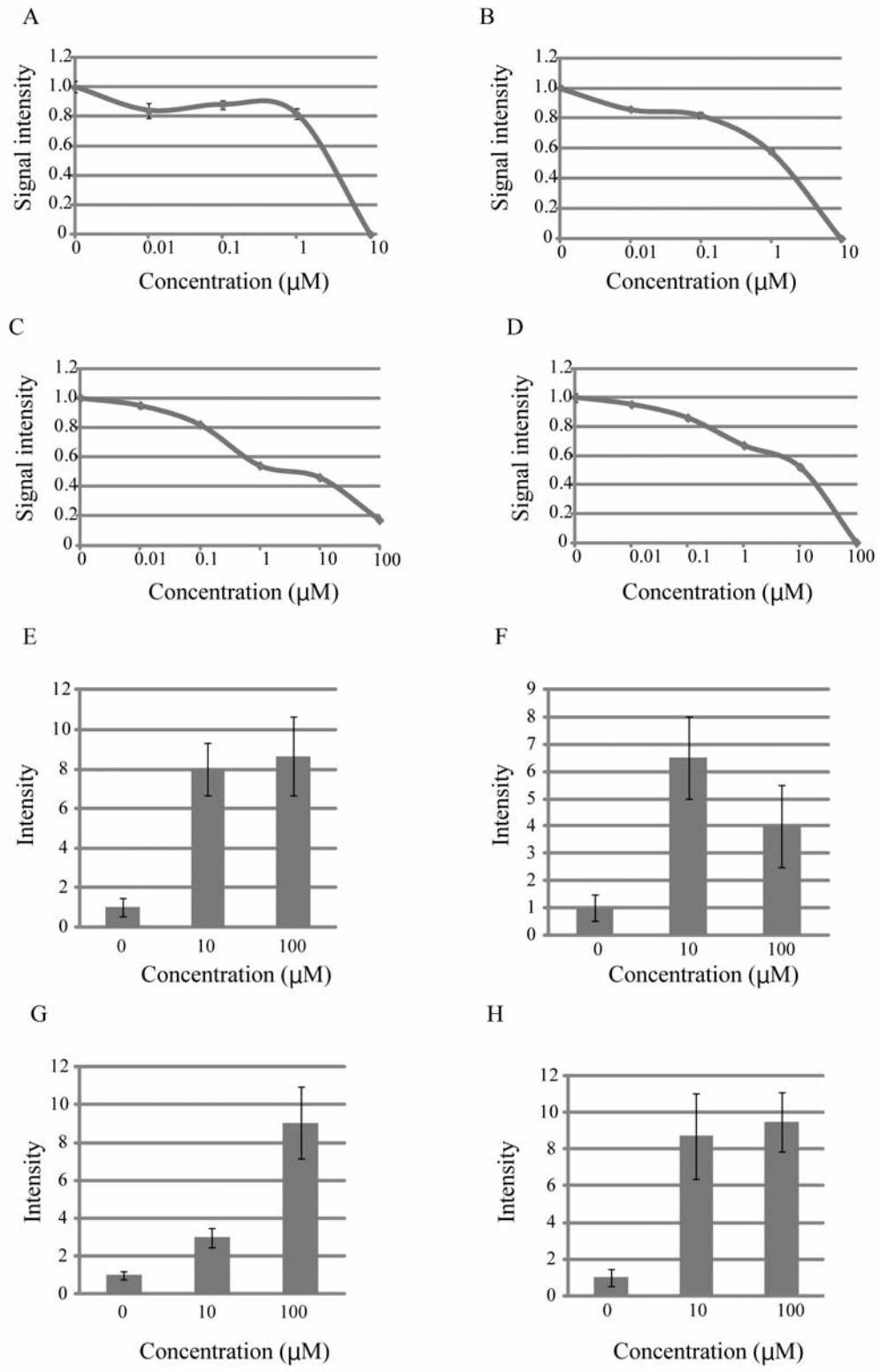

Figure 3. *CS-1 (A and C) and SW1353 (B and D) cells were treated with the indicated concentration of ABT-263 (A and B) or ABT-737 (C and D) for 72 h. The signal intensities by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, indicating cell viability, were normalized using the intensity of the untreated cells. CS-1 (E and G) and SW1353 (F and H) cells were treated with the indicated concentration of ABT-263 (E and F) or ABT-737 (G and H) for 48 h. The intensities indicating the apoptosis rate by ELISA were normalized using the intensity for the untreated cells.*

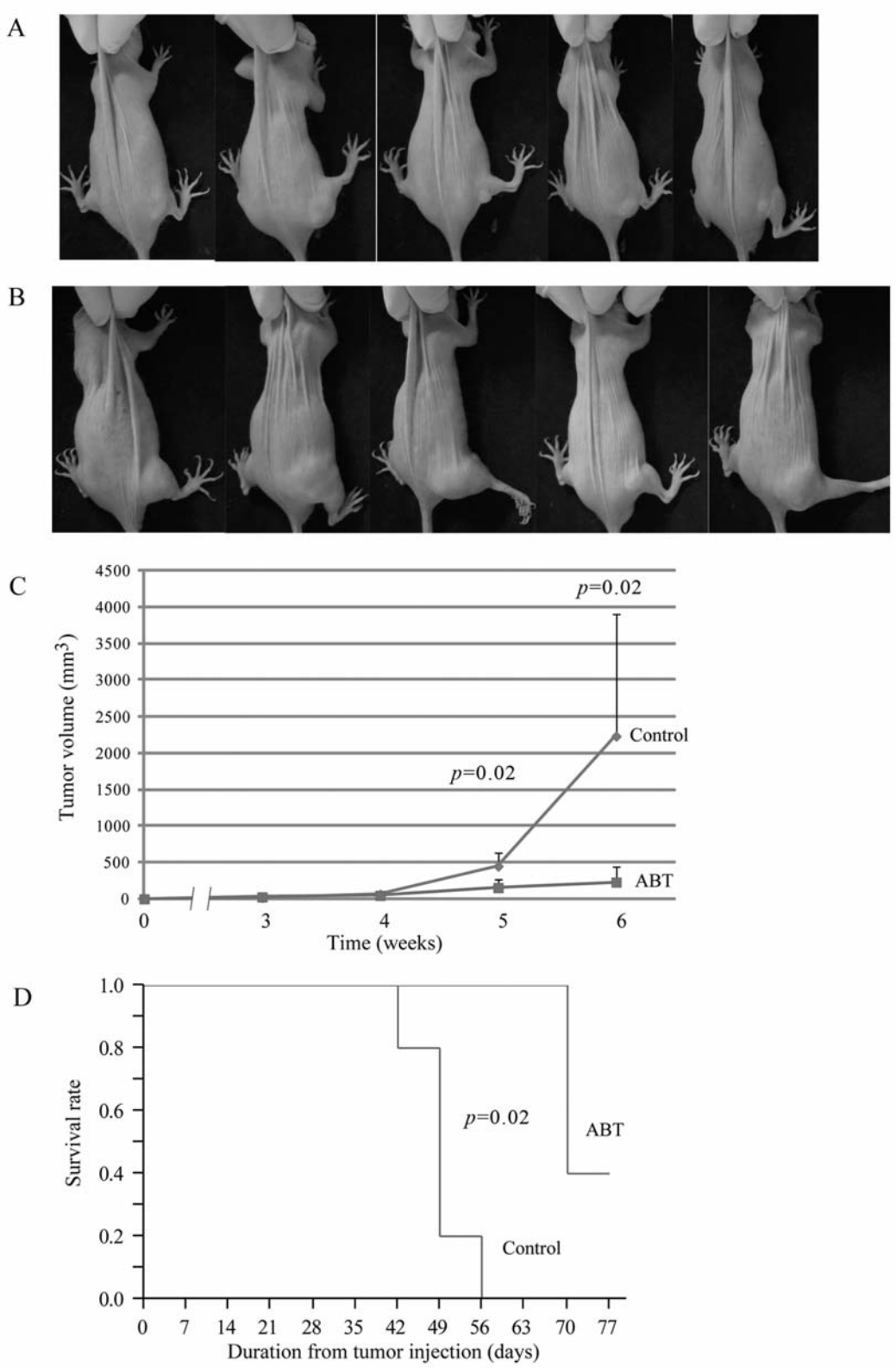

Figure 4. *In vivo effect of ABT-263 on chondrosarcoma cell growth. Macroscopic findings in a chondrosarcoma mouse model 6 weeks after subcutaneous inoculation of SW1353 cells into the right hind leg with (A) and without (B) ABT-263 treatment. C: Tumor volumes of animals treated or not with ABT-263. Significant growth inhibition by ABT-263 was confirmed 5 and 6 weeks after tumor injection. D: Kaplan–Meier curves show significantly prolonged survival in the treated group (p=0.002, log-rank test).*

that ABT-737 alone could not control chondrosarcoma growth *in vitro* (11). They reported an half maximal inhibitory concentration (IC_{50}) of 20-135 μM in 2-5×10^4 cells per well in 96-well plates. We do not know why our result, which suggests the possibility of growth inhibition by a single BH3 mimetic, differs from that of van Oosterwijk *et al*. The studies differed in the cell lines used, cell density, and agent concentrations. Van Oosterwijk *et al*. used more cells *in vitro*. Cell density seemed to have a large effect on the biological efficacy of the tested chemical agents. In fact, they used about 10-times more cells than were used in our study. We found diminished effect of BH3 mimetics on cell growth with increased cell numbers in the MTS assay (data not shown). Their intent may have been to confirm the cytotoxic effect on established chondrosarcoma rather than on tumor growth inhibition. In the clinical setting, these agents could be used continuously for more prolonged duration such as two to three weeks; thus, these *in vitro* studies provide only a modest portrayal of the effect of the agent on growth inhibition. Thus if they used the parameters we did, the IC_{50} of the BH3 mimetics might have been smaller in their study.

More importantly, we used an *in vivo* chondrosarcoma animal model for the first time, suggesting the possibility of growth inhibition and significant improvement of survival with the administration of a clinically-available concentration of BH3 mimetic ABT-263 alone *in vivo*. Hence we believe that at least for growth inhibition *in vitro* and *in vivo*, but not for cytotoxic effect of the established lesion, a single BH3 mimetic could have some effect.

We were unable to demonstrate the relative efficacy of ABT-737 and ABT-263 in controlling the growth of chondrosarcoma. Although the IC_{50} of ABT-263 was found to be significantly lower compared to that of ABT-737, macroscopic analysis revealed no striking difference in growth inhibition. Only ABT-263 can be administered *per os*, which is an advantage; however, the biological properties and administration schedules should be determined for these promising agents.

In conclusion, BH3 mimetics inhibited growth of chondrosarcoma by inducing apoptosis and are promising candidates for adjuvant therapy.

Conflicts of Interests

The Authors declare that they have no conflicts of interest.

Acknowledgements

We are grateful to Ms. Mizuho Kosuge and Ms. Miyuki Murayama for their skilful technical assistance. This study was supported in part by the Health and Labour Sciences Research Expenses for Commission, Applied Research for Innovative Treatment of Cancer from the Ministry of Health, Labor and Welfare H26-084) and by a Research Encouragement Award by Kyorin University School of Medicine.

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Received August 12, 2014
Revised August 21, 2014
Accepted August 25, 2014