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3-Hydroxy-3′,4′-dimethoxyflavone suppresses Bcl-w-induced invasive potentials and stemness in glioblastoma multiforme

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ABSTRACT

3-Hydroxy-3′,4′-dimethoxyflavone (HDMF) is a natural chemical product that is not currently regarded as a drug. In our study, we employed glioblastoma cells and cell biology and biochemistry approaches to investigate the potential of HDMF as a natural anticancer therapy option. FACS analysis showed that treatment concentration of HDMF does not exert cytotoxicity on U251 cells. Wound-healing and invasion assays showed that HDMF dose-dependently decreased the migratory and invasive potentials of these cells, likely by indirectly inhibiting MMP-3 activity as a result of the inhibition of p38 and ERK signaling proteins – an effect of HDMF also shown by Western blotting. HDMF inhibits Bcl-w-induced neurosphere formation and the expression of glioma stem cell markers, such as Musashi, Sox-2 and c-myc. These results indicate that HDMF suppresses migratory or invasive potentials and stemness and functions as a negative agent against the aggressiveness of glioblastoma cells. We propose that HDMF has potential as anticancer drug for inhibiting the aggressiveness of glioblastoma multiforme (GBM).

1. Introduction

The Natural Product Collection, available from MicroSource (Discovery Systems), is a source of 800 naturally occurring compounds that are supplied in a readily purified form. These compounds include the flavonoid, 3-hydroxy-3′,4′- dimethoxyflavone (HDMF), which has attracted some attention due to its potential therapeutic properties; HDMF has been shown to exhibit antioxidant properties, potential estrogenic activity, and anticancer activities, such as antiproliferative effects, and the promotion of differentiation and apoptosis [1–3]. However, despite these properties, the medicinal benefits of this natural compound remain to be evaluated.

Glioblastoma multiforme (GBM) is the most common and aggressive human primary brain tumor and is also known as a glioma as it arises from glial cells [4,5]. GBM is difficult to treat using the conventional therapeutic options, such as standard surgical resection, radiation and chemotherapy, owing to its high frequency of recurrence [6,7]. Additionally, glioma cells are highly proliferative and exhibit mesenchymal characteristics [8,9], leading to tumor progression through acquisition of invasive or metastatic potential [6,7,5] or a stem cell-like phenotype [9–11]; these processes have been linked to the upregulation of the prosurvival and antiapoptotic protein, B cell lymphoma-w (Bcl-w) [12]. Bcl-w is expressed in a variety of cancer types, including GBM [8,13] and colorectal adenocarcinomas [14,15], as well as gastric cancers [16,17], and our previous data have shown that Bcl-w expression is positively associated with the invasiveness or stemness of both gastric cancer cells [16,17] and GBM [8,9].

In this study, to assess the possible anticancer properties of HDMF, we treated U251 or U87MG cells, which constitute a model cell line for GBM, with HDMF and assessed the resulting migratory and invasive potentials or stemness of the cells. Furthermore, for evaluating the effects of HDMF on U251 or U87MG cells, we used a Bcl-w-induced migration and invasion model, and to assess the action of HDMF at the molecular level, we used Western blotting.

2. Materials and methods

2.1. Cell culture and transfection

U251 cells were obtained from the Korean Cell Line Bank (KCLB). Cells were cultured in Minimum Essential Medium Eagle

Abbreviations: HDMF, 3-hydroxy-3′,4′-dimethoxyflavone; GBM, glioblastoma multiforme; Bcl-w, B cell lymphoma-w; MMP-3, Matrix metalloproteinase-3; MMP-9, Matrix metalloproteinase-9.

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(Cellgro Mediatech, Inc., Manassas, VA, USA) medium containing 10% FBS, 0.1% penicillin–streptomycin (PAA Laboratories GmbH, Pasching, Austria). For functional analyses, cells were transiently transfected with the vector control or Bcl-w-overexpression constructs by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation.

2.2. Antibodies and treatments

The antibodies were used as follows; anti-p-p38, anti-p38, anti-p-ERK, anti-ERK, anti-vimentin, anti-Bcl-w and anti-Musashi were purchased from Cell Signaling technology (Beverly, MA, USA). Anti-cdc42, anti-MMP-3 and anti-c-myc were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin was obtained from Sigma–Aldrich (St Louis, MO). Anti-MMP-9 was purchased from Calbiochem (La Jolla, CA). Anti-Sox-2 was purchased from R&D Systems (Minneapolis, MN).

The Natural Product Collection has 800 compounds. 3-hydroy-3′,4′-dimethoxyflavone (HDMF) is one of these compounds which were purchased from MicroSource, Discovery Systems (Gaylordsville, CT). HDMF was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM and stored in aliquots at −20 °C. Cells were treated with HDMF at a final concentration of 10 μM.

2.3. Western blot analysis

Proteins either in conditioned media or in cell lysates resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After the transfer, the membranes were blocked in 5% skim milk in Tris-buffered saline containing Tween 20 (TBST) for 1 h, and subsequently blotted using the indicated antibodies in 5% skim milk in TBST for 2 h at room temperature. The membranes were washed 3 times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody followed by using an enhanced chemiluminescence (ECL) detection system (Amersham, Uppsala, Sweden).

2.4. FACS analysis

Cell death assessed using PI (propidium iodide) staining followed by flow cytometry. Control or treated cells were harvested in cold PBS and collected by centrifugation for 10 min at 3000 rpm. Cells were incubated in PI staining solution (5 ng/mL) for 5 min at room temperature. Cells (10,000 per sample) were analyzed on a FACS scan flow cytometer (Becton Dickinson, NJ). Data was analyzed by Cell Quest software.

2.5. Wound healing assay

The confluent monolayer cell was scratched using a tip and then allowed to migrated for 16–24 h at 37 °C. the cells were washed twice with PBS, and fixed with methanol : acetic acid (3:1) for 30 min followed by staining with 1% crystal violet in 10% ethanol for 30 min. Cell in five fields in the scratched area (200 × 500 μm^2 area) were counted under a light microscope (Mitoti AE31 series, Trinocular inverted MIC). Results were analyzed for statistical significance using Student's *t* test. Differences were considered significant at $p < 0.05$.

2.6. Matrigel invasion assay

These assays were conducted as described previously [12] . In brief, to compare invasiveness, cells (2×10^5) in 200 μl of medium were seeded onto the upper surfaces of Matrigel-coated polycarbonate filters which were placed in modified Boyden chambers

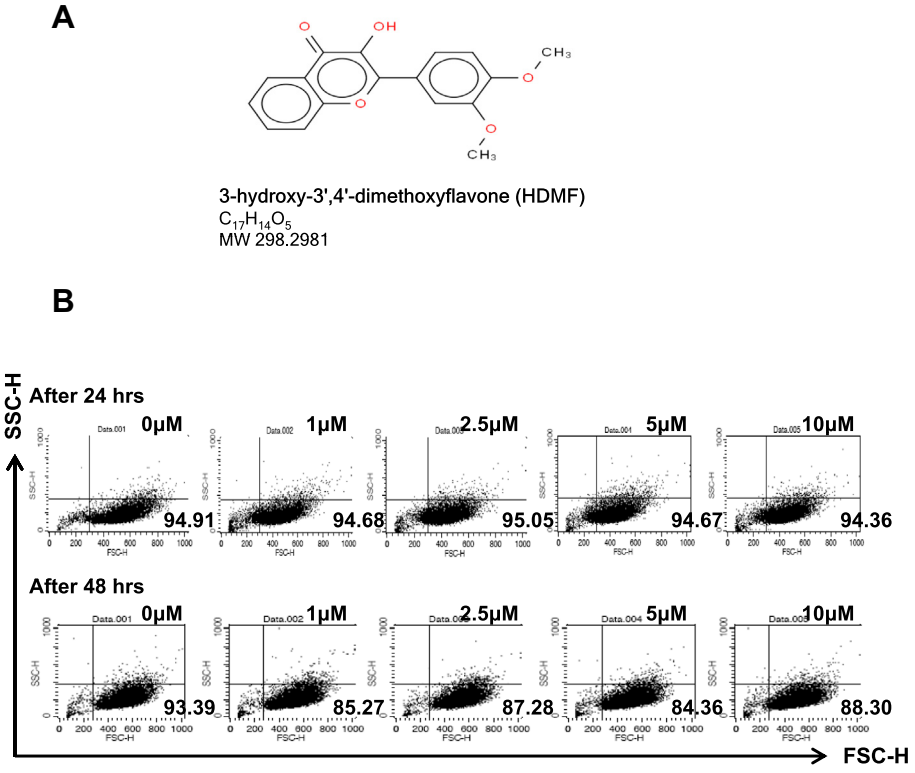

Fig. 1. Characteristics of 3-hydroxy-3′,4′-dimethoxyflavonol (HDMF). (A) The structure of HDMF is shown. (B) HDMF (1–10 μM) does not exhibit cytotoxicity in U251 cells, as shown by FACS analysis. Cell death was assessed using propidium iodide (PI) staining followed by flow cytometry. For this, cells were resuspended in PI solution (5 μg/mL) and analyzed for PI staining using a FACS scan flow cytometer. Data was then analyzed using the Cell Quest software.

(Corning, NY) that contain ECM components (BD Biosciences, Bedford, MA). The lower compartments of the chambers were filled with 1 ml of serum-free media supplemented with 0.1% BSA. After 20–24 h of incubation at 37 °C, the cells that had migrated to the lower surface of the filter were fixed and stained using a Diff-Quick kit (Fisher Scientific, Pittsburgh, PA), then counted under a microscope (Mitoti AE31 series, Trinocular inverted MIC). The results were analyzed for statistical significance using the Student's *t*-test. Differences were considered to be significant at $p < 0.05$.

2.7. Neurospheres culture

The vector control or the Bcl-w overexpressing U251 or U87MG cells were suspended in Dulbecco's modified Eagle's medium-F12 (Cellgro, Manassas, VA) containing 20 ng/ml each of epidermal growth factor (EGF, Biovision, Milpitas, CA), basic fibroblast growth factor (bFGF, Biovision, Milpitas, CA) and B27 (1:50) (GIBCO, Langley, OK) as a stem-cell-permissive medium. Spheres were harvested after 2–4 days and protein extracts were subject to Western blotting with appropriate antibodies.

2.8. Neurospheres formation assay

For generating spheres formation, sphere cells were dissociated with Accutase (Innovative Cell Technologies, Inc). Spheres-forming cells were distributed into 96-well plates at a density of 1–2 cells per well. After 12 h, individual wells were visually checked for the presence of a single cell. After 1, 10 and 20 days until the sphere formation, spheres were attached by 10% FBS and stained with Coomassie Brilliant Blue R-250 solution (bioWORLD, Dublin, OH). Sphere with a diameter >20 μm were counted under an inverted microscope using Miotic AE31 series.

2.9. Statistical analysis

Statistical significance was assessed using a Student's *t*-test. A *p* value $^{*}p < 0.05$ $^{**}p < 0.005$ $^{***}p < 0.0005$ compare with the control was considered statistically significant.

3. Results

3.1. HDMF does not exhibit cytotoxicity in U251 cells

The Natural Product Collection compound, HDMF (structure shown in Fig. 1A), was tested for cytotoxic effects on U251 cells. For this, we applied HDMF at concentrations within the ranges of 1–10 μM, for 24 or 48 h, which was followed by FACS analysis of the cells. The data clearly show that none of the HDMF applications resulted in cytotoxicity in U251 cells, and therefore that HDMF does not affect the cell viability of U251 cells (Fig. 1B).

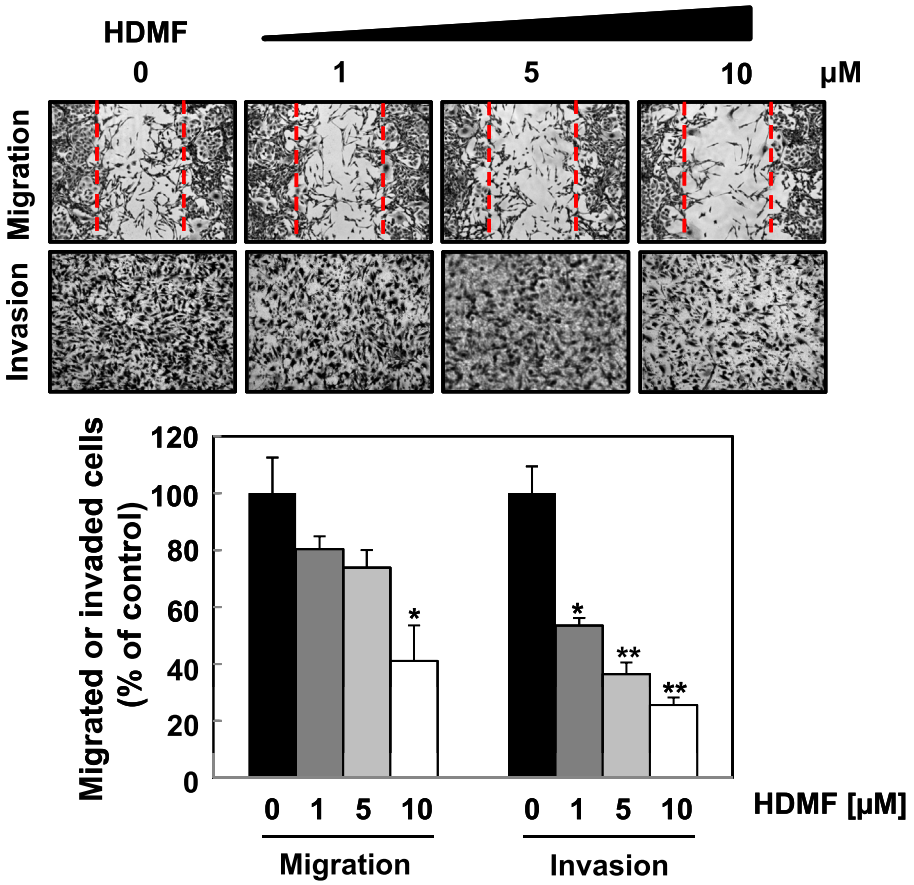

Fig. 2. HDMF inhibits the cell motility and invasiveness of U251 cells in a dose-dependent manner. Following treatment of U251 cells with HDMF (0, 1, 5, 10 μM) for 16 h, a marked decrease in the migration and invasion potentials of U251 cells was seen using the wound-healing assay and Matrigel invasion assay, respectively, and these decreases were dose dependent. Analysis of the invasive potential of U251 cells was carried out using Matrigel-coated polycarbonate filters. For this, cells were incubated for 20–24 h in modified Boyden chambers and cells penetrating the filters were stained and counted under a light microscope. Experiments were performed in triplicate, and means from control and HDMF experiments were found to be significantly different. $^{*}p < 0.05$; $^{**}p < 0.005$.

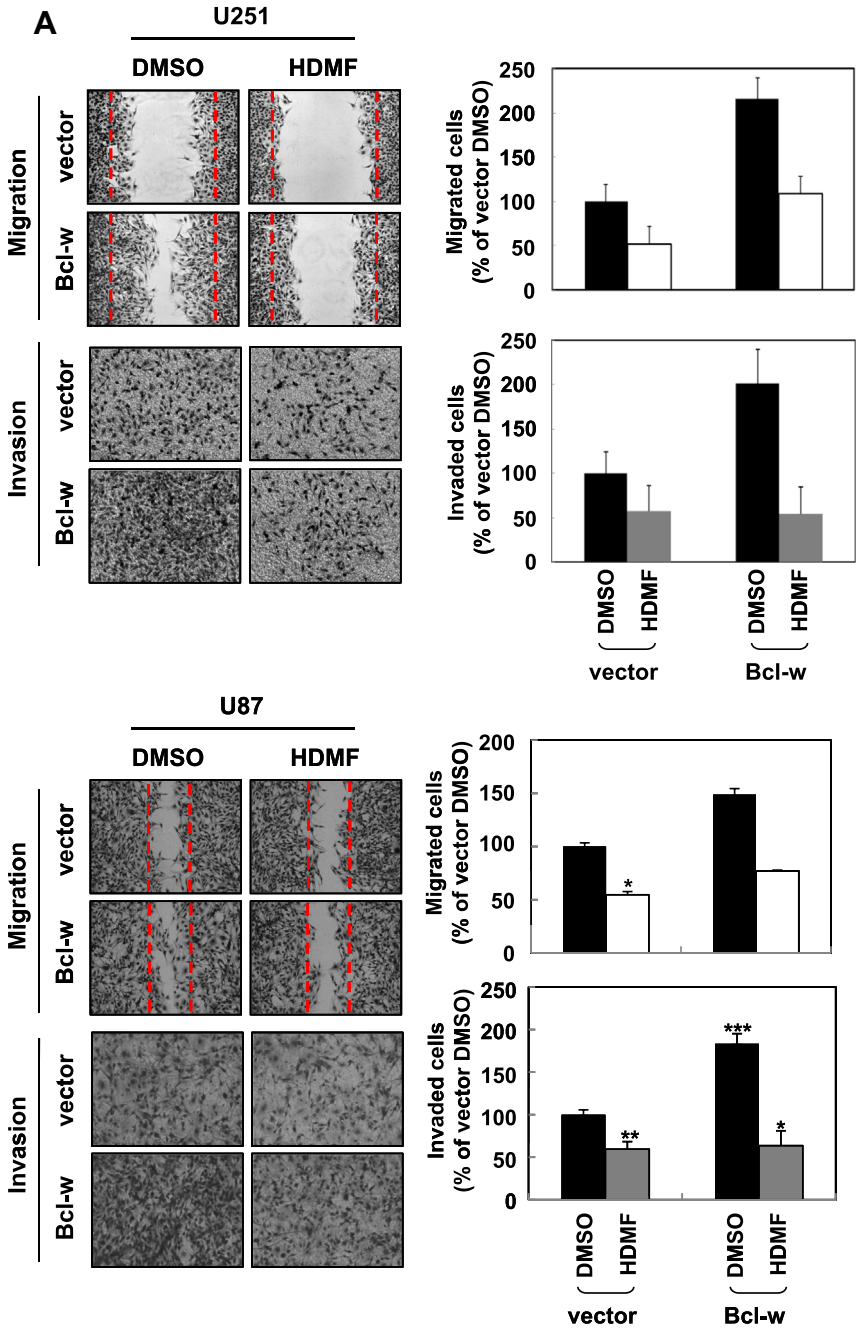

Fig. 3. HDMF negatively regulates Bcl-w-induced migration and invasion by reducing vimentin, cdc42 or MMP-3 expression, through inactivation of the p38 and ERK signaling pathways. (A) U251 or U87MG cells were transfected with either an empty vector (control) or Bcl-w cDNA. The migratory or invasive potential of U251 or U87MG cells was analyzed using wound-healing assay or Matrigel-coated polycarbonate filters, respectively. Cells were incubated for 20–24 h in modified Boyden chambers, and cells penetrating the filters were stained and counted under a light microscope. Experiments were performed in triplicate, and means from control and HDMF experiments were found to be significantly different. $^{**}p < 0.005$. (B) Control or Bcl-w-overexpressing U251 or U87MG cells were treated with HDMF (10 μM) for 1 h, after which they were subjected to Western blotting with appropriate antibodies (such as p-p38, p38, p-ERK, ERK, vimentin, Cdc42, Bcl-w, MMP-3 and MMP-9). Anti-beta-actin or Ponceau S staining was performed as a loading control, respectively.

3.2. HDMF suppresses migration and invasion of U251 glioblastoma cells

Following the treatment of U251 (Fig. 2) or U87MG (data not shown) cells with either DMSO (vehicle control) or HDMF (0, 1, 5 and 10 μM), the cells were assessed for migratory and invasive potentials using the wound-healing and Matrigel invasion assays. The results showed that both potentials were markedly decreased in HDMF-treated cells.

3.3. HDMF negatively regulates Bcl-w-induced migration and invasion by reducing vimentin, cdc42 or MMP-3 expression through inactivation of the p38 and ERK signaling pathways in U251 or U87MG cells

Data from the wound-healing and the Matrigel invasion assays showed that HDMF significantly impairs the Bcl-w-induced migration and invasion potentials of glioblastoma cells (Fig. 3A). Control or Bcl-w-overexpressing U251 or U87MG cells were treated with

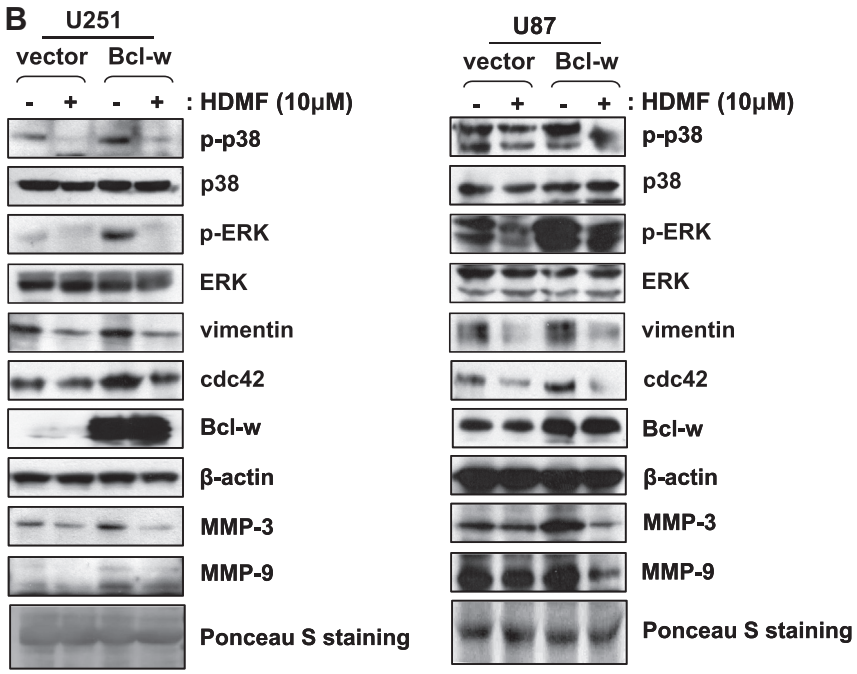

Fig. 3 (*continued*)

HDMF (10 μM) for 1 h and were subjected to Western blotting with appropriate antibodies (Fig. 3B). The Western blotting data showed that the expression of the MAPK proteins p38 and ERK in their phosphorylated forms (p-p38 and p-ERK) decreased in HDMF-treated cells; vimentin, cdc42, MMP-3 or MMP-9 expression also decreased. Therefore, to explain the significant inhibition of the Bcl-w-induced migration and invasion potentials of U251 or U87MG cells by HDMF, it could be speculated that HDMF indirectly decreases MMP-3 expression in these cells through dephosphorylation and inactivation of p38 and ERK MAPK signaling proteins.

3.4. HDMF downregulates the neurospheres formation of U251 or U87MG cells and reduces the expression levels of glioma stem-like cell markers

Neurospheres-forming assays have been widely used to retrospectively evaluate the stemness of cells, i.e., their capacity for self-renewal and differentiation, both of which are instrumental in cancer cell formation. Therefore, it was of interest to evaluate the effects of HDMF on Bcl-w-induced neurospheres formation in U251 or U87MG cells. As shown in Fig. 4A, Bcl-w-induced neurospheres formation ability was decreased by treatment with HDMF. Additionally, the expression levels of the Bcl-w-induced stem cells markers Musashi, Sox2 and c-myc, were all significantly decreased by treatment with HDMF (Fig. 4B). Therefore, taken together, this evidence suggests that HDMF negatively regulates Bcl-w-induced stemness.

4. Discussion

Promising findings from our own studies and those of other studies related to the antiproliferative effects of the naturally occurring flavonoid [1,2] , HDMF, prompted us to investigate the potential antitumor properties of this compound against glioblastoma cells, through a combination of cell biology and biochemical approaches, employing U251 or U87MG glioblastoma cells as a model cell line. Through FACS analysis we found that this compound did not confer cytotoxic effects on the U251 cells, as cell viability was maintained at all the concentrations tested (0, 1, 5 and 10 μM; Fig. 1B). HDMF treatment resulted in a marked dose-dependent decrease in the migration and invasion potentials of U251 cells, as was found using the wound-healing and Matrigel invasion assays, respectively (Fig. 2).

Our previous studies showed that the Bcl-w protein increased the migratory or invasive potentials and stemness, of U251 cells [8,9] ; therefore, to confirm the above effects of HDMF, we applied this Bcl-w-induced aggressiveness model to our analyses on the anticancer effects of HDMF. We found that 10 μM of HDMF significantly suppressed the Bcl-w-induced migration and invasiveness of U251 or U87MG cells (Fig. 3A). Since this suppression was concurrent with the inhibition of both MMP-3 expression and phosphorylation of p38 and p-ERK (Fig. 3B), we suggest that suppression of migration and invasiveness could be explained by the decreased vimentin, cdc42 and MMP-3 expression, which was caused by the inhibition of p38 and ERK. Others studies that vimentin or MMP-3 is key regulator of epithelial–mesenchymal transition (EMT) in various cancers [18] and highly expressed in glioblastoma cells [19] are supported our data. In addition, we found that treatment with HDMF reduced neurospheres formation of U251 or U87MG cells (Fig. 4A) and decreased the Bcl-w-induced expression of the stem cell markers Musashi, Sox2 and c-myc in these cells (Fig. 4B). Taken together, it can be concluded that HDMF negatively regulates the Bcl-w-induced aggressiveness of U251 or U87MG cells.

In the present study, we have shown that by inhibiting Bcl-w-induced actions, HDMF functions as a negative agent against the aggressiveness of glioblastoma cells. On the basis of the results of this study, we propose that an anticancer treatment using the naturally occurring compound, HDMF, could specifically target the properties of migrating, invasive, or cancer stem-like cells, thus avoiding damage to normal cells. Taken together, this compound could represent a novel clinical strategy for targeting glioblastomas.

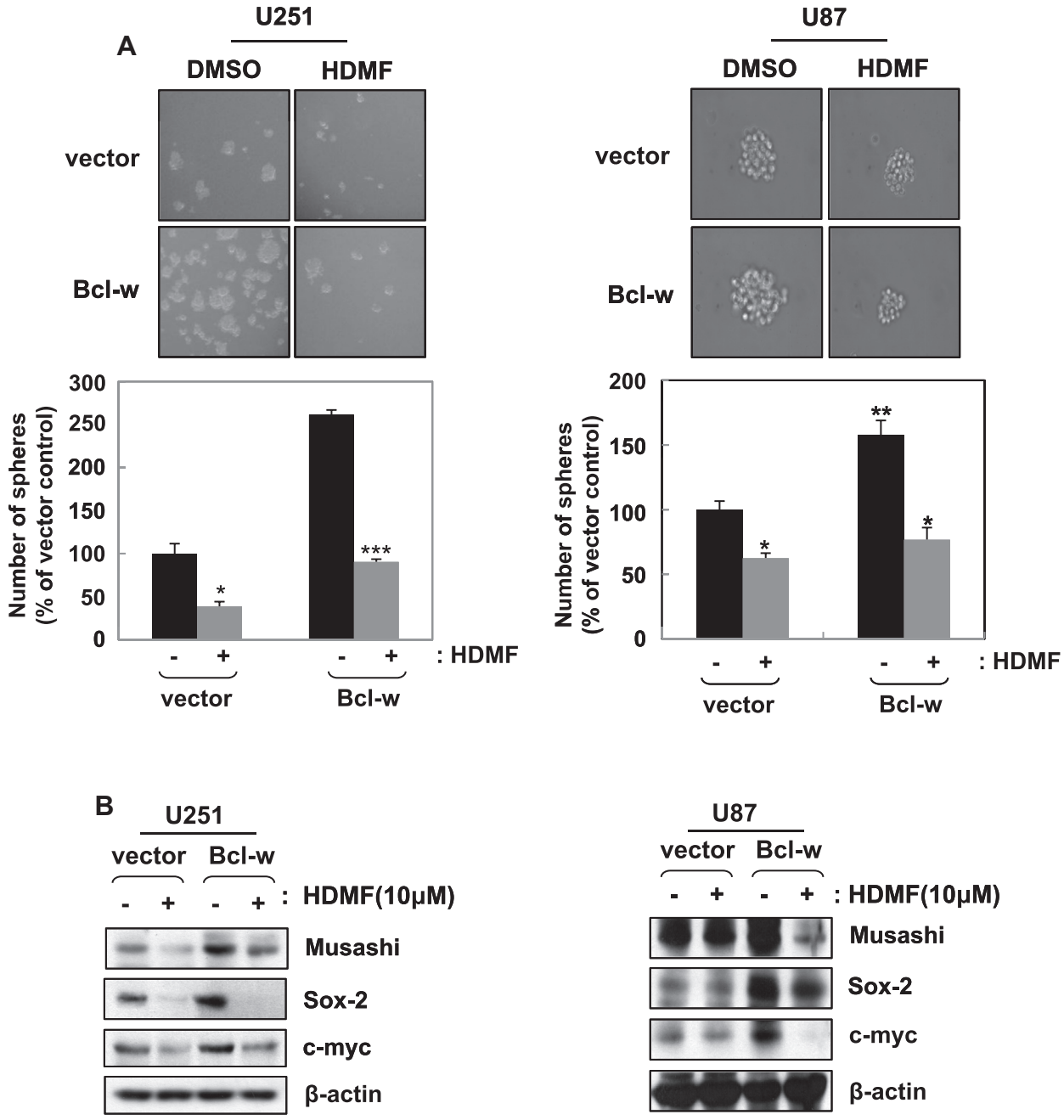

Fig. 4. HDMF downregulates the neurospheres formation of U251 glioma cells and suppresses expression of stemness-related transcription factors. (A) Data show the results of a neurosphere-formation assay that was performed on DMSO- or HDMF-treated cells. $^{*}p < 0.05$; $^{***}p < 0.0005$, $n = 5$. (B) The expression of the glioma tumor-initiating cell markers, Musashi, Sox2 and c-myc were compared using Western blotting. Beta-actin was used as a loading control.

Disclosure statement

The authors have no conflict of interest.

Acknowledgments

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