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ORIGINAL ARTICLE

The HDAC inhibitor OBP-801 suppresses the growth of myxofibrosarcoma cells

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Summary

Purpose: *Myxofibrosarcoma is characterized by a high rate of recurrence after surgery. Since myxofibrosarcoma is refractory to conventional cytotoxic chemotherapy, the established radical treatment is primary wide resection. The effects of histone deacetylase (HDAC) inhibitors on myxofibrosarcoma have not yet been investigated. Therefore, the main purpose of the present study was to examine the effects of a HDAC inhibitor on myxofibrosarcoma.*

Methods: *The effects of the HDAC inhibitor OBP-801 on human myxofibrosarcoma cells were examined using cell viability assay, flow cytometric analysis of the cell cycle and apoptosis, and Western blotting. The effects of combinations of OBP-801 with pazopanib or Akt-mTOR inhibitors were also investigated using cell viability assay.*

Results: *OBP-801 inhibited the growth of myxofibrosarcoma NMFH-1 and NMFH-2 cells. It also induced cell cycle arrest at the G2 phase and apoptosis in both cell lines. The inhibitory effects of pazopanib and Akt-mTOR inhibitors on the growth of myxofibrosarcoma cells were enhanced by the combination with OBP-801.*

Conclusions: *The present results demonstrated that OBP-801 exerted therapeutic effects in myxofibrosarcoma in both single and concomitant administrations. Therefore, OBP-801 has potential as a novel treatment for myxofibrosarcoma.*

Key words: *HDAC inhibitor, myxofibrosarcoma, G2 arrest, apoptosis*

Introduction

There are more than 50 different types of soft tissue sarcomas. Myxofibrosarcoma is an adult sarcoma occurring in extremities, and one of the aggressive types of soft tissue neoplasms. It is recognized as a distinct fibroblastic neoplasm that is characterized by a myxoid nodular appearance and curvilinear vasculature with a considerably broad spectrum of nuclear pleomorphisms, cellularity, and mitosis [1]. Enzinger and Weiss described the myxoid variant of malignant fibrous histiocytoma [2], and Angervall and Kindblom were the first to use the term myxofibrosarcoma [3]. Myxofibrosarcoma was included as a separate entity in the 2002 WHO classification [4], and its histological grades are classified according to the French Federation of Cancer Centers (FNCLCC) grading system [5].

Myxofibrosarcoma is clinically characterized by high rates of local recurrence [6] and metastasis [7,8], with increases with the histological grade [9]. However, promising treatments have been limited to complete surgical resection, and thus, alternative treatments are desired. Soft tissue sarcomas, including myxofibrosarcoma, are comparatively uncommon diseases and comprise more than 50

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subtypes. Thus, advances in new therapies for soft tissue sarcoma have been delayed [10].

The multi-targeted tyrosine kinase inhibitor pazopanib was approved for sarcoma, based on the findings of the PALETTE study [11]. Trabectedin was subsequently approved in many countries for the treatment of advanced soft tissue sarcomas [12,13]. Although clinical trials have not yet been conducted on all subtypes, pazopanib and trabectedin have been approved for all subtypes of soft tissue sarcomas, due to their rarity. Many other medicines and chemicals have been identified as novel therapeutic agents and undergone clinical trials. In the present study, we focused on the efficacy of the histone deacetylase (HDAC) inhibitor OBP-801 against myxofibrosarcoma.

HDAC inhibitors are chemical compounds that have been attracting interest as a novel targeted therapy for various malignant tumors [14-16]. HDACs have been shown to promote cell proliferation and are regarded as a therapeutic target in sarcomas [17]. Clinical trials have been conducted on HDAC inhibitors as a single agent or in combination with other agents for the treatment of sarcomas [18-22]. The rarity of each sarcoma and diversity of subtypes are factors contributing to the difficulties associated with assessing the effectiveness of candidate agents [16]. Although myxofibrosarcoma is comparatively a common subtype of adult sarcoma, candidate agents, including HDAC inhibitors, have not yet been evaluated in detail.

We herein for the first time demonstrated that the HDAC inhibitor OBP-801 inhibited the growth of myxofibrosarcoma cells by G2/M cell cycle arrest and apoptosis with the induction of p21 and several proapoptotic molecules.

We also showed that the combination of OBP-801 with pazopanib or Akt-mTOR inhibitors more effectively suppressed cell growth than each agent alone. Collectively, the present results indicate the potential of OBP-801 as a candidate therapeutic agent for myxofibrosarcoma.

Methods

Reagents

OBP-801 (Oncolys Biopharma, Tokyo, Japan), pazopanib (Chem Scene, NJ, USA), temsirolimus (Sigma, St Louis, MO, USA), NVP-BEZ235 (Selleck Chemicals, TX, USA), and z-VAD-fmk (R&D Systems, MN, USA) were dissolved in dimethyl sulfoxide (DMSO) and stored at -20 ºC. The final DMSO concentration of each reagent was maintained at 0.1% for all samples.

Cell culture

Human myxofibrosarcoma NMFH-1 cells were purchased from the RIKEN BioResource Center (BRC, Tsukuba, Japan), and NMFH-2 cells were established from the primary tumor of a patient with myxofibrosarcoma by Hiroyuki Kawashima. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin in an incubator at 37ºC with 5% CO_2. Cells from passages three to six were used in the experiments.

Cell viability assay

Myxofibrosarcoma cells were seeded on 96-well plates and incubated with the indicated concentrations of various agents for 72 h for NMFH-1 cells or for 48 h for NMFH-2 cells. The number of viable cells was assessed using the Cell Counting Kit-8 assay according to the manufacturer's instructions (Dojindo Molecular Technology, Kumamoto, Japan). The kit reagent was added to the medium and incubated for a further 4 h. The absorbance of samples at 450 nm was measured with a multi-well spectrophotometer, and cell viability was analyzed.

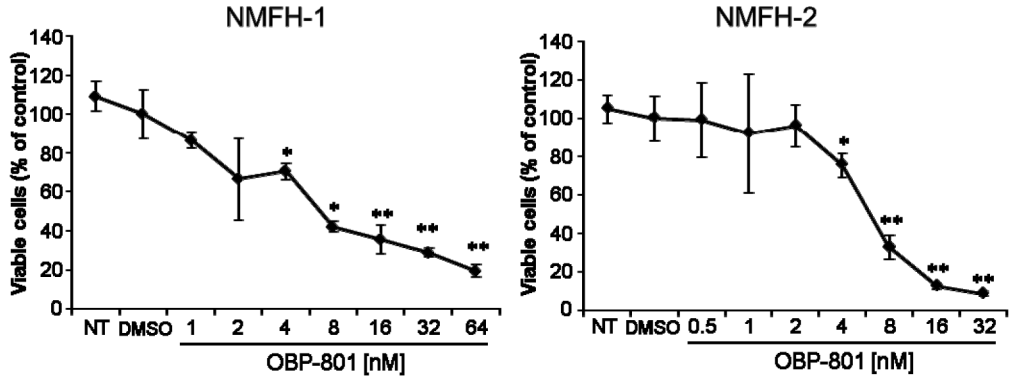

Figure 1. Effects of OBP-801 on the growth of myxofibrosarcoma cells. Human myxofibrosarcoma NMFH-1 and NMFH-2 cells were treated with the indicated concentrations of OBP-801. After incubation for 72 h for NMFH-1 cells or for 48 h for NMFH-2 cells, viable cell numbers were evaluated using Cell Counting Kit-8. The Figure shows that OBP-801 suppressed the growth of myxofibrosarcoma cells. Significance was compared with DMSO treatment control (n=3;*p<0.05, **p<0.01 vs control).

Analysis of cell cycle progression

Myxofibrosarcoma cells were seeded in 6-well plates and incubated with the indicated concentrations of various agents for 72 h for NMFH-1 cells or for 48 h for NMFH-2 cells. Myxofibrosarcoma cells were harvested and washed with phosphate-buffered saline (PBS), and suspended in PBS containing 0.1% TritonX-100 and 10 μg/ml propidium iodide (PI) (SIGMA). A flow cytometric analysis was performed with 10,000 cells using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). CellQuest software (Becton Dickinson) and the ModFit LT V2.0 software package (Verity Software House, Topsham, ME, USA) were used for all analyses.

Western blot analysis

Cultured cells were rinsed with PBS, lysed in lysis buffer (50 mM Tris-HCl, 1% SDS, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM PMSF, and 1 mM DTT), and sonicated. Lysates were centrifuged at 15,000 rpm for 20 min, and supernatants were collected. Supernatants

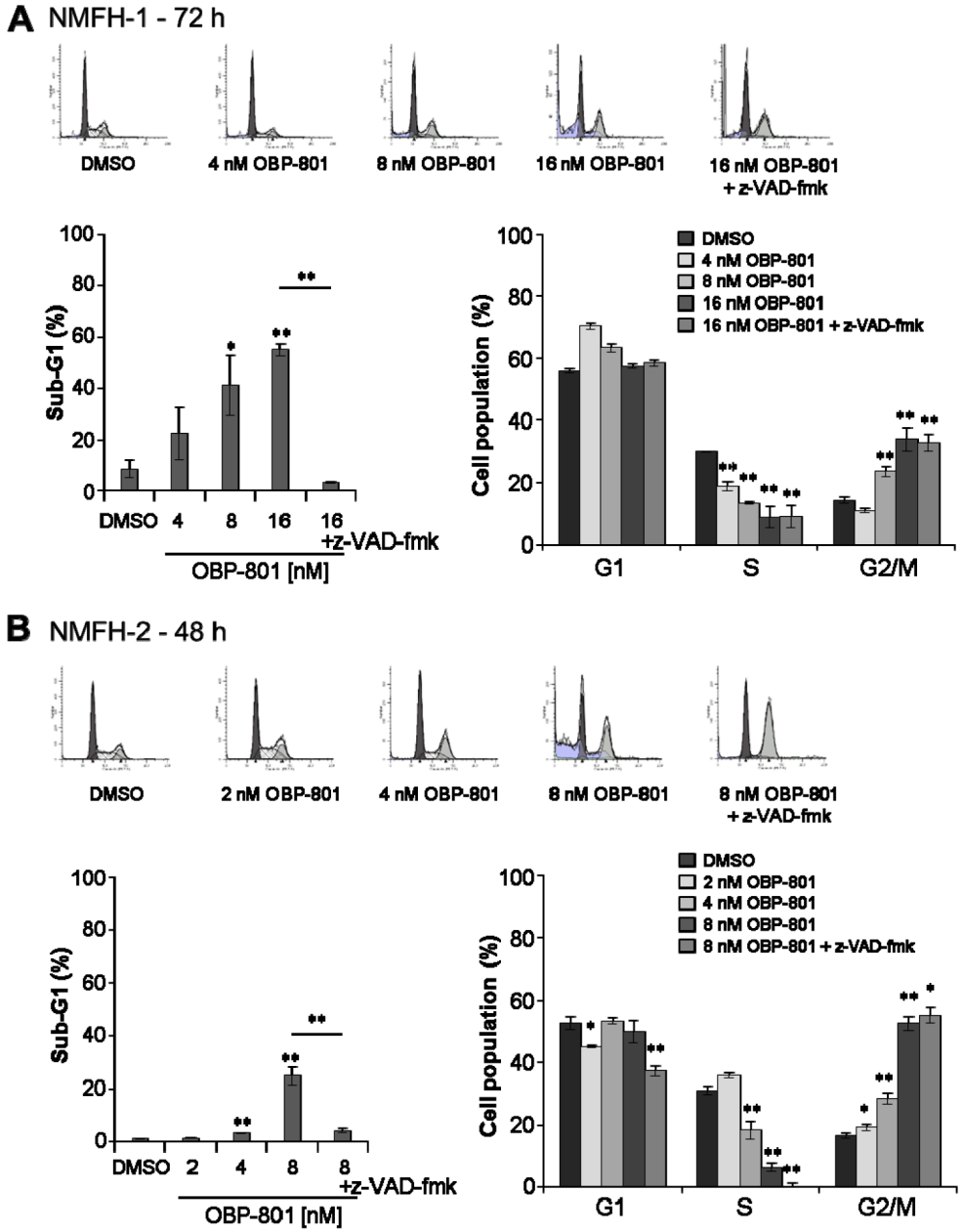

Figure 2. Effects of OBP-801 on cell cycle progression in myxofibrosarcoma cells assessed by flow cytometry. Data are shown as representative cell cycle histograms, sub-G1 populations and the proportions of cells in the G1, S, and G2/M phases of the cell cycle. **A:** NMFH-1 cells were treated with the indicated concentrations of OBP-801 with or without the pan-caspase inhibitor z-VAD-fmk for 72 h. The Figure shows that OBP-801 induced C2/M cell cycle arrest and induced apoptosis after incubation for 72 h in NMFH-1 cells. Significance was compared with a DMSO treatment control (n=3;*p<0.05, **p<0.01 vs control). **B:** NMFH-2 cells were treated with the indicated concentrations of OBP-801 with or without the pan-caspase inhibitor z-VAD-fmk for 48 h. The Figure shows that OBP-801 induced C2/M cell cycle arrest and induced apoptosis after incubation for 48 h in NMFH-2 cells. Significance was compared with a DMSO treatment control (n=3;*p<0.05, **p<0.01 vs control).

were subjected to SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes, and analyzed by immunoblotting. An anti-Bax antibody was purchased from BD Biosciences (San Hose, CA, USA). An anti-GAPDH antibody was purchased from HYTEST (Turku, Finland). Anti-Bad, anti-phospho-histone H3 (Tyr705), anti-histone H3, anti-acetyl-histone H3 (Lys9), anti-p21$^{Waf1/Cip1}$, and anti-Bim antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). An anti-Bcl-2 antibody was purchased from Abcam (Cambridge, UK). An anti-survivin antibody was purchased from R&D systems. An anti-β-actin antibody was purchased from Sigma. Blots were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare, Piscataway, NJ, USA). Signals were detected using a Chemilumi-one chemiluminescent kit (Nacalai Tesque, Kyoto, Japan) and an Immobilon Western Chemiluminescent HRP Substrate (Millipore, Burlington, MA, USA).

Statistics

All statistical tests were performed with EXCEL software (Microsoft).

Data are presented as mean ± standard deviations (SD). All experiments were performed in triplicate for each drug concentration. Data were analyzed using the Student's *t*-test and differences were considered to be significant at $p<0.05$.

Results

OBP-801 suppressed the growth of myxofibrosarcoma cells

To investigate growth inhibition by OBP-801 in myxofibrosarcoma cells, we assessed viable cell numbers after the OBP-801 treatment. NMFH-1 and NMFH-2 cells were incubated with the indicated concentrations of OBP-801. After incubation for 72 h for NMFH-1 cells or for 48 h for NMFH-2 cells, viable cell numbers were evaluated. As shown in Figure 1, OBP-801 suppressed the growth of both cell lines in a dose-dependent manner. We also examined the effects of OBP-801 on cell cycle progression and apoptosis using a flow cytometric analysis. As shown

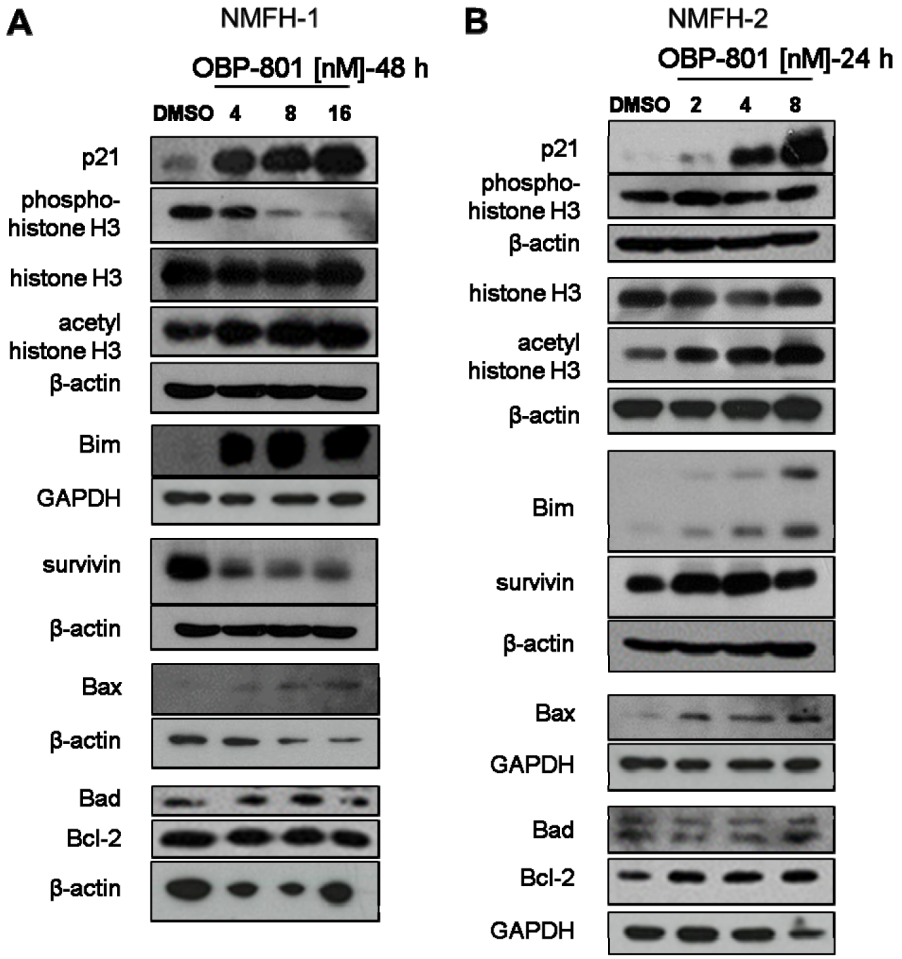

Figure 3. The effects of OBP-801 on expression of phospho-histone H3, histone H3, acetyl-histone H3, p21, Bim, Bax, Bad, Bcl-2, and survivin detected via western blotting. GAPDH and β-actin were loading controls. **A:** NMFH-1 cells were treated with the indicated concentrations of OBP-801 for 48 h and showed the induction of p21, acetyl-histone H3, Bim and Bax, and the reduction of phospho-histone H3 and survivin. **B:** NMFH-2 cells were treated with the indicated concentrations of OBP-801 for 24 h and showed the induction of p21, acetyl-histone H3, Bim and Bax.

in Figure 2, OBP-801 induced G2/M cell cycle arrest in NMFH-1 and NMFH-2 cells. OBP-801 dose-dependently induced apoptosis for 72 h in NMFH-1 cells and for 48 h in NMFH-2 cells. The pan-caspase inhibitor z-VAD-fmk blocked the induction of apoptosis by OBP-801 in both myxofibrosarcoma cell lines. These results showed OBP-801 inhibited cell growth by G2/M cell cycle arrest and caspase-dependent apoptosis in NMFH-1 and NMFH-2 cells.

OBP-801 induced G2 phase arrest in myxofibrosarcoma cells

To confirm that OBP-801 inhibited HDAC activities, acetylated histone H3 was investigated using Western blotting. OBP-801 enhanced the acetylation of histone H3 after the treatment for 24 h in NMFH-1 and NMFH-2 cells (Figure 3).

To clarify whether OBP-801 induced G2 or M phase arrest, we examined the phosphorylation of

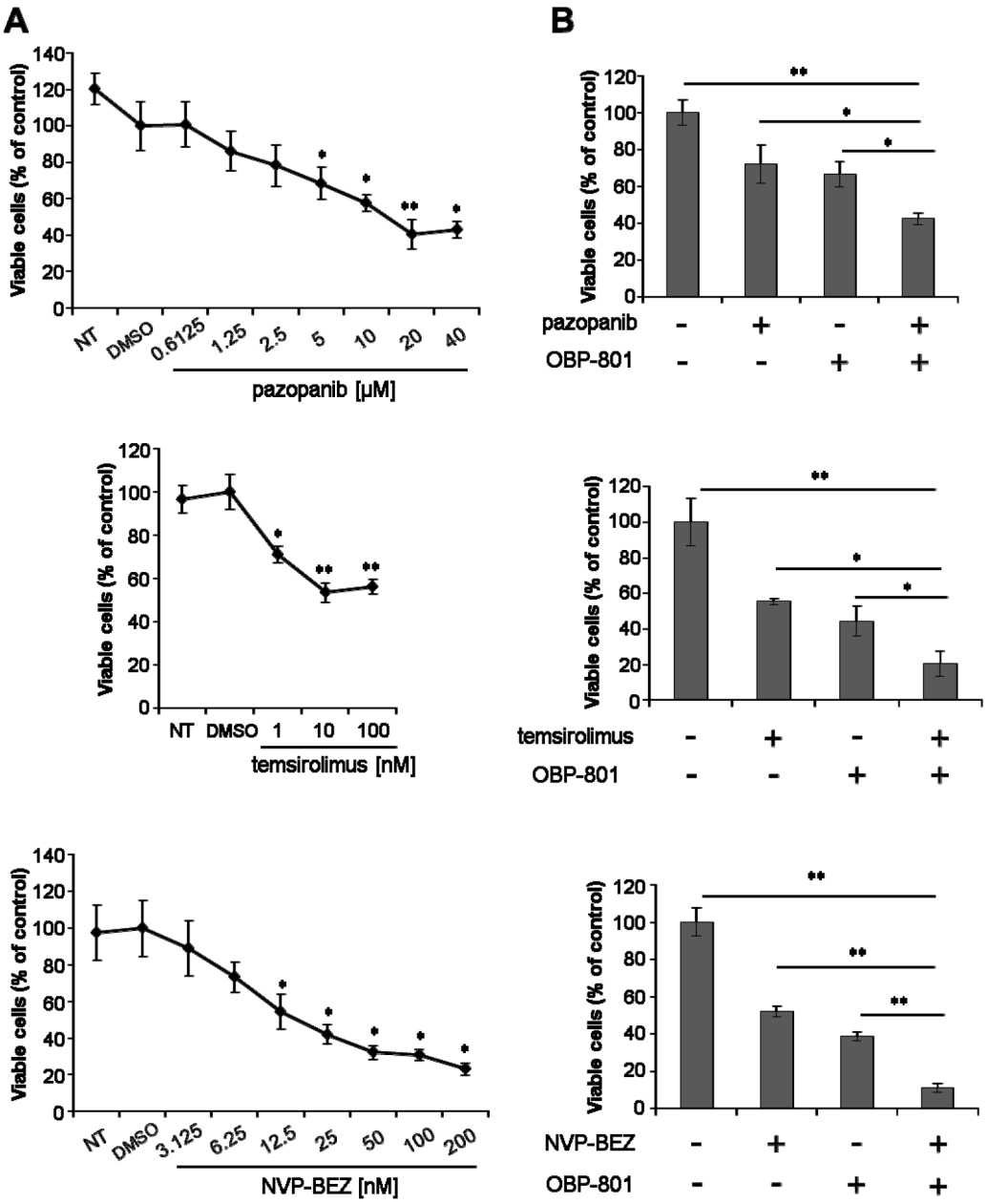

Figure 4. Effects of pazopanib, temsirolimus, and NVP-BEZ on the growth of myxofibrosarcoma NMFH-1 cells. **A:** NMFH-1 cells were treated with the indicated concentrations of each chemical agent. After incubation for 72 h, viable cell numbers were evaluated using Cell Counting Kit-8. The Figure shows that Pazopanib, Temsirolimus and NVP-BEZ inhibited cell growth of NMFH-1 cells in a dose-dependent manner. Significance was compared with a DMSO treatment control (n=3;*p<0.05, **p<0.01 vs control). **B:** NMFH-1 cells were treated with the indicated concentrations of 8 nM OBP-801 and/or 10 μM pazopanib, 100 nM temsirolimus, and 50 nM NVP-BEZ. After incubation for 72 h, viable cell numbers were evaluated using Cell Counting Kit-8. The Figure shows that OBP-801 enhances these inhibitory effects more than each agent alone. Significance was compared with a DMSO treatment control (n=3; *p<0.05, **p<0.01 vs. control).

histone H3 (Ser10) as one of the M phase markers. The phosphorylation of histone H3 was decreased by the treatment with OBP-801 for 48 h in NMFH-1 cells. In NMFH-2 cells, the phosphorylation of histone H3 was not significantly altered by the treatment for 24 h. These results suggest that OBP-801 caused G2 cell cycle arrest in both myxofibrosarcoma cell lines. OBP-801 markedly increased the expression of p21 in NMFH-1 and NMFH-2 cells.

Effects of OBP-801 on the expression of proapoptotic and antiapoptotic molecules in myxofibrosarcoma cells

Among the proapoptotic molecules examined, Bim and Bax were increased by the OBP-801 treatment in both myxofibrosarcoma cell lines (Figure 3). Bad was not increased by the OBP-801 treatment. On the other hand, the expression of one of the antiapoptotic members, Bcl-2, was not significantly affected by OBP-801 in both myxofibrosarcoma cell lines. Survivin functions to inhibit the activation of caspase, leading to the negative regulation of apoptosis. The expression of survivin was reduced by the OBP-801 treatment in NMFH-1 cells.

OBP-801 exerts synergistic effects with pazopanib and other mTOR inhibitors to suppress the growth of NMFH-1 cells

Pazopanib has been approved for the treatment of soft tissue sarcomas. Akt-mTOR pathway inhibitors are expected to exert therapeutic effects against myxofibrosarcoma [23]. We examined the effects of the mTOR inhibitor temsirolimus and the dual PI3K/mTOR inhibitor NVP-BEZ235 in NMFH-1 cells. Viable NMFH-1 cell numbers after the treatment with the different concentrations of agents were assessed. As shown in Figure 4A, pazopanib, temsirolimus, and NVP-BEZ235 inhibited cell growth in a dose-dependent manner. Furthermore, the co-treatment of each agent with OBP-801 enhanced these inhibitory effects more than each agent alone, as shown as Figure 4B.

Discussion

HDAC inhibitors are promising new agents for the treatment of malignant neoplasms, such as multiple myeloma and cutaneous T cell lymphomas [13]. Previous studies demonstrated that cancer development and its persistence could be induced by changes in epigenetic modifications [13-15]. The acetylation and deacetylation of lysine residues on histone proteins modify chromatin strands and regulate gene expression by transcription factors. OBP-801 is a cyclic peptide-based HDAC inhibitor, similar to trapoxin and romidepsin (Istodax).

In the present study, we investigated the effects of OBP-801 on human myxofibrosarcoma NMFH-1 and NMFH-2 cells. The results obtained demonstrated that OBP-801 exerted inhibitory effects on growth in both cell lines by inducing G2 cell cycle arrest and apoptosis.

G2/M cell cycle arrest through the induction of p21 by HDAC inhibitors has been reported in various cancer cell lines [24,25]. In the present study, the HDAC inhibitor OBP-801 induced cell cycle arrest at the G2 phase in both myxofibrosarcoma cell lines (Figures 2,3). Our results revealed the induction of p21 as one of the mechanisms responsible for G2 cell cycle arrest by OBP-801 (Figure 3). A previous study reported that p21 inhibited Cdc2/cyclin B1 complex and G2/M transition [26]. On the other hand, HDAC inhibitors were shown to activate the Sp1 site- and/or p53 site-dependent transcription of p21 [27,28].

The induction of apoptosis by HDAC inhibitors was previously shown to contribute to their effects in many cancer models *in vitro* and *in vivo* [29-31]. HDAC inhibitors may induce apoptosis through extrinsic and intrinsic pathways against sarcomas, and also enhance the susceptibility of osteosarcoma cells to apoptosis. In the present study, we examined whether apoptosis induced by OBP-801 was caspase-dependent using the pan-caspase inhibitor z-VAD-fmk. The results obtained showed that the pan-caspase inhibitor markedly inhibited apoptosis in both myxofibrosarcoma cell lines (Figure 2). We then investigated various molecules related to caspase-dependent apoptosis. Bcl-2 is one of the antiapoptotic molecules in the Bcl-2 family. OBP-801 did not significantly affect the expression of Bcl-2, but induced the expression of the proapoptotic proteins, Bim and Bax (Figure 3).

Bim has been identified as a key molecule of apoptosis by HDAC inhibitors in several cancer cells, including fibrosarcoma HT1080 cells [32]. The knockdown of Bim by siRNA was shown to suppress apoptosis by the HDAC inhibitor, trichostatin A (TSA) [33]. Furthermore, the relationship between Bim and other apoptotic molecules has been examined in several studies. Bim is a member of the proapoptotic Bcl-2 homology 3 (BH3)-only family of proteins. Once BH3-only proteins neutralize antiapoptotic Bcl-2 family members, proapoptotic Bax/Bak is activated and translocates to mitochondria [34]. In the present study, OBP-801 strongly induced Bim in both myxofibrosarcoma cell lines (Figure 3). Bax and Bim were also induced by OBP-801. Furthermore, the expression of the antiapoptotic protein survivin was reduced by OBP-801 in NMFH-1 cells, but not in NMFH-2 cells (Figure 3).

We herein demonstrated for the first time the

potential of a HDAC inhibitor as an effective treatment for myxofibrosarcoma. In previous studies, HDAC inhibitors exerted inhibitory effects on cell growth in other sarcomas, such as liposarcoma [35], synovial sarcoma [36], rhabdomyosarcoma [37], or osteosarcoma [38]. The present results showed that OBP-801 induced G2 cell cycle arrest and apoptosis in myxofibrosarcoma cells as well as in other sarcoma cells. In rhabdomyosarcoma cells, the HDAC inhibitor SAHA up-regulated p21 expression and induced cell cycle arrest [37]. In undifferentiated pleomorphic sarcoma, a treatment with a HDAC inhibitor caused G2/M cell cycle arrest and apoptosis with the induction of p21 and Bim [39]. The mechanisms underlying cell cycle arrest and apoptosis may be common among sarcomas.

Clinical trials on HDAC inhibitors have been undertaken to assess their antitumor therapeutic efficacies against soft tissue sarcomas [18]. Furthermore, several clinical trials have been conducted to evaluate the efficacy of HDAC inhibitors both as monotherapy and in combination with standard chemotherapeutic agents or targeted therapeutic drugs for sarcomas [17]. Combination therapy of a HDAC inhibitor with another molecular targeting agent is expected in order to achieve an acceptable toxicity profile [15].

In the present study, the combination of OBP-801 with pazopanib suppressed the growth of myxofibrosarcoma cells (Figure 4B). Pazopanib has been approved for the treatment of soft tissue sarcomas. However, in clinical trials on pazopanib in Japanese patients with myxofibrosarcoma, 6/8 patients achieved stable disease [40]. Based on the present results and previous findings [19,41], HDAC inhibitors are promising candidates in combined treatments with pazopanib. In addition, the activation of the Akt/mTOR pathway has been reported in myxofibrosarcoma [23]. Therefore, we also examined the combined effects of OBP-801 with the mTOR inhibitor temsirolimus and/or dual PI3K/mTOR inhibitor NVP-BEZ *in vitro*. The results obtained showed that the combined treatment of OBP-801 with temsirolimus and/or NVP-BEZ more effectively reduced the growth of myxofibrosarcoma cells (Figure 4B).

In conclusion, the HDAC inhibitor OBP-801 may enhance the effects of pazopanib and Akt/mTOR inhibitors in the treatment of myxofibrosarcoma. Myxofibrosarcoma is a rare sarcoma characterized by multiple local recurrences and an optimal treatment strategy has not yet been established. The present results suggest the potential of OBP-801 as a promising agent for the treatment of myxofibrosarcoma.

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Conflict of interests

The authors declare no conflict of interests.

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