The Effects of Histone Deacetylase Inhibitors on the Induction of Differentiation in Chondrosarcoma Cells

Riku Sakimura, Kazuhiro Tanaka, Syunsaku Yamamoto, Tomoya Matsunobu, Xu Li, Masuo Hanada, Takamitsu Okada, Tomoyuki Nakamura, Yang Li, and Yukihide Iwamoto

Abstract

Purpose: Histologically, chondrosarcomas represent the degree of chondrogenic differentiation, which is associated with the prognosis of the disease. Histone acetylation and deacetylation play key roles in the regulation of chondrocytic differentiation. Here, we describe the antitumor effects of histone deacetylase (HDAC) inhibitors as differentiating reagents on chondrosarcomas.

Experimental Design: We examined the effects of a HDAC inhibitor, depsipeptide, on the growth of chondrosarcoma cell lines. We also investigated the modulation of the expression levels of extracellular matrix genes and the induction of phenotypic change in chondrosarcoma cells treated with depsipeptide. Finally, we examined the antitumor effect of depsipeptide on chondrosarcoma in vivo.

Results: Dipsipeptide inhibited the growth of chondrosarcoma cells by inducing cell cycle arrest and/or apoptosis. HDAC inhibitors increased the expression of the α1 chain of type II collagen (COL2A1) gene due to the enhanced histone acetylation in the promoter and enhancer. Dipsipeptide also up-regulated the expressions of aggrecan and the α2 chain of type XI collagen (COL11A2) mRNA in a dose-dependent manner. Moreover, long-term treatment with a low dose of depsipeptide resulted in the induction of differentiation into hypertrophic phenotype, as shown by the increment of the α1 chain of type X collagen (COL10A1) expression in chondrosarcoma cells. In vivo studies and histologic analyses confirmed that depsipeptide significantly inhibited tumor growth and induced differentiation into the hypertrophic and mineralized state in chondrosarcoma cells.

Conclusions: These results strongly suggest that HDAC inhibitors may be promising reagents for use as a differentiating chemotherapy against chondrosarcomas.

Chondrosarcomas represent the second most prevalent primary skeletal malignancy. Because chondrosarcomas are highly resistant to conventional chemotherapy and radiotherapy, surgery is the only effective form of treatment. To date, no effective adjuvant therapy exists for inoperable chondrosarcomas (1, 2). Thus, a novel approach to therapeutic strategy is a priority for improving the prognosis as related to local recurrence, metastasis, and survival.

A recent study has shown that the cartilaginous cells of the growth plate during their different states of differentiation and the distinct histologic chondrosarcoma subtypes show striking histologic similarities (3). The normal growth plate represents the chondrocytic maturation process including resting, proliferative, prehypertrophic, and hypertrophic chondrocytes. As a final step, the cartilage matrix becomes mineralized and hypertrophic cells undergo apoptosis. The process is accompanied by the sequential gene expression pattern of the characteristic extracellular matrix. After commitment to the chondrocyte lineage, mesenchymal cells undergo condensation, cease to express type I collagen (COL1), and then differentiate into a chondrocytic phenotype characterized by the expression of type II, IX, and XI collagens (COL2, COL9, and COL11), as well as aggrecan. Hypertrophic chondrocytes are marked by their unique gene product, type X collagen (COL10). Histochemical and immunohistochemical analyses have shown that chondrosarcomas showing mainly mature and terminally differentiated (hypertrophic) chondrocytic phenotypes display only scant proliferation, whereas less differentiated chondrosarcomas with the phenotype of dedifferentiated chondrocytes show significantly higher proliferative activity (4). These features are highly correlated with prognosis (5). These findings suggest the possibility that the induction of differentiation in chondrosarcoma cells could lead to a novel therapeutic strategy.

Recently, several studies have shown that the transcriptional regulating effects of histone acetylation or deacetylation...
play important roles in specific gene expressions of chondrocytes (6–9). A report has also shown that treatment with the histone deacetylase (HDAC) inhibitor trichostatin A enhances the expression of the α1 chain of type II collagen (COL2A1) and aggrecan in human primary chondrocytes (10). HDAC inhibitors represent a novel class of antineoplastic agents and several HDAC inhibitors are currently under clinical trial (11). A number of studies have shown that HDAC inhibitors cause a variety of phenotypic changes, such as cell cycle arrest, morphologic reversion of transformed cells, apoptosis, and differentiation (12, 13). In this study, we report the antitumor activity of a HDAC inhibitor, depsipeptide (FK228, FR901228), for chondrosarcomas both in vitro and in vivo. We investigated the possibility that HDAC inhibitors might have a role in cellular differentiation of chondrosarcomas. This is the first report showing the potential clinical usefulness of HDAC inhibitors in the treatment of chondrosarcomas.

Materials and Methods

Cells and reagents. SW1353, OUMS-27, and RCS cells were cultured as previously described (14–16). Depsipeptide was generously provided by Gloucester Pharmaceuticals, Inc. (Cambridge, MA). Depsipeptide was dissolved in 100% ethanol at a concentration of 1 mg/mL and stored at −20°C until use.

Cell viability assay. The cell viability assay was carried out as previously described (17) using CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI). Luminescence was measured using Microlumat Plus LB 96V (EG&G, Berthold, Germany).

Western blot. Western blot analyses were carried out as previously described (18) with antibodies against human p21Waf1/Cip1 (BD PharMingen, San Diego, CA), and acetylated histone H3 (Upstate Biotechnology, Waltham, MA).

Flow cytometry. Flow cytometric analyses were done as previously described (13).

RNA extraction and quantitative real-time reverse transcription-PCR (TaqMan PCR). RNA extraction and quantitative real-time reverse transcription-PCR were carried out as previously described (13). The amplification primers and fluorogenic hybridization probes were designed using Primer Express 1.0 (PE Applied Biosystems, Foster City, CA). The sequences of the primers and TaqMan probes are shown in Table 1.

Transfection and luciferase assay. The transfection and luciferase assays using reporter constructs, pKN185luc and pKN159luc, were done as previously described (19). We used pGL2-Basic vector and pRL-SV40 as negative control and internal control for normalization of transfection efficiency, respectively.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were carried out as previously described (20). The primers used to amplify the enhancer region have previously been described (21). The following primers were also used: Promoter, 5′-ACCTCAGAGAGG-3′ and 5′-CAGCCGCTCTGGGCTTCT-3′; 5′-flank, 5′-GAGCGGCTATGCCCCGAGAGC-3′ and 5′-TTATAACGGAGG-3′.

In vivo antitumor activity of depsipeptide. RCS cells were harvested from the monolayer culture at 80% confluency, resuspended in DMEM at 8 × 10^6 viable cells/mL, mixed with a collagen gel to form a 1:1 volume, and then s.c. inoculated into 5-week-old female athymic nu/nu mice (1 × 10^6 viable cells per mouse). Growth of s.c. tumors became evident after 14 days, and then the mice were grouped into three of six each (day 0). Depsipeptide suspended in PBS was administered i.p. at a total dose of 0, 5, or 10 mg/kg in six evenly divided doses on days 0, 4, 8, 14, 18, and 22. Mice in the control group were injected with PBS only. Mice were followed up every 3 to 4 days by measurement of the body mass, as well as the length and width of each tumor. The tumor weight was calculated according to the method described by Ueda et al. (22). Multivariate analysis was done by ANOVA. For analysis of significance, Fisher’s protected least significant difference test was used. Treatment-related toxicity was evaluated by serial weight measurements and gross anatomy. On day 25, the mice were killed and the tumors were resected and subjected to histologic evaluation. All the animal experiments were done in accordance with the Institutional Guidelines for Animal Experiments of Kyushu University.

Histologic and immunohistochemical analyses. Tumors were fixed with 10% (v/v) formalin in 0.9% NaCl solution before paraffin embedding and routine sectioning for H&E, safranin O, Alizarin red, and immunohistochemistry stains. The synthesis or depletion of glycosaminoglycans was visualized by safranin O staining (23). To detect mineralization, Alizarin red staining was done according to the method of Dahl (24). To investigate alkaline phosphatase activity, the tumor samples were frozen in optimum cutting temperature compound and cryosectioned. The tissue sections were fixed with acetone at 4°C for 5 min and then subjected to alkaline phosphatase staining using the azo dye technique (25). Immunohistochemical staining was also done as previously described (26) with an antibody against COL10 (>100; Calbiochem, San Diego, CA).

Results

Depsipeptide inhibits the growth of chondrosarcoma cells by inducing cell cycle arrest and apoptosis. At first, the effects of a HDAC inhibitor, depsipeptide, on the growth of chondrosarcoma cell lines were examined. As shown in Fig. 1A, depsipeptide inhibited the proliferation of the chondrosarcoma cells in a dose-dependent manner. All of the cell lines tested were sensitive to depsipeptide in the nanomolar range with mean IC_{50} values of 0.69 to 4.07 nmol/L. HDAC inhibition by depsipeptide leads to an accumulation of acetylated nucleosome core histones and transcriptional activation of a subset of genes including, most consistently, the cyclin-dependent kinase inhibitor p21Waf1/Cip1. Western blot analyses showed a dose-dependent induction of p21Waf1/Cip1 expression and the acetylation of histone H3 by treatment with depsipeptide in

<table>
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<th>Reverse</th>
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accumulation of a cell population within the sub-G1 fraction, the cell cycle. Higher doses of depsipeptide resulted in the confirmation that depsipeptide dose-dependently increased the induction of COL2A1 mRNA expression in chondrosarcoma cells. To examine whether or not depsipeptide could induce COL2A1 expression in chondrosarcoma cells. OUMS-27 cells were treated with depsipeptide for 24 h and analyzed for the expressions of p21Waf1/Cip1 and acetylated histone H3 (Ac-H3) by Western blot analyses. The effect of depsipeptide is shown as a percentage of ATP luminescence relative to the control. Points, mean of three independent experiments; bars, SE.

HDAC inhibitors modulate the expressions of extracellular matrix genes in chondrosarcoma cells. We examined whether or not depsipeptide could also modulate the expression levels of other extracellular matrix genes in chondrosarcoma cells. Treatment with 0.925 nmol/L depsipeptide, a relatively low dose, for 21 days and the expression levels of the extracellular matrix genes were investigated using TaqMan PCR. In SW1353 cells treated with depsipeptide, the mRNA expressions of COL2A1, aggregan, and COL11A2 were increased until 4 or 7 days after the treatment, and then they decreased moderately (Fig. 4A-C). As shown in Supplementary Fig. S1, other HDAC inhibitors, apicidin and trichostatin A, showed similar effects on the modulation of the extracellular matrix gene expressions in chondrosarcoma cells. These data indicated that HDAC inhibitors might induce chondrocytic maturation in chondrosarcoma cells.

Depsipeptide induces differentiation into a hypertrophic phenotype in chondrosarcoma cells. Next, we did time-course experiments. SW1353 and OUMS-27 cells were treated with 1.85 nmol/L depsipeptide, a relatively low dose, for 21 days and the expression levels of the extracellular matrix genes were investigated using TaqMan PCR. In SW1353 cells treated with depsipeptide, the mRNA expressions of COL2A1, aggregan, and COL11A2 were increased until 4 or 7 days after the treatment, and then they decreased moderately (Fig. 4A-C, SW1353). The expression level of COL10A1 mRNA was slightly induced (Fig. 3C). On the other hand, COL1A1 mRNA was decreased by the treatment with 3.70 to 14.8 nmol/L of depsipeptide (Fig. 3D). As shown in Supplementary Fig. S1, other HDAC inhibitors, apicidin and trichostatin A, showed similar effects on the modulation of the extracellular matrix gene expressions in chondrosarcoma cells. These data indicated that HDAC inhibitors might induce chondrocytic maturation in chondrosarcoma cells.
Fig. 2. Induction of COL2A1 expression in chondrosarcoma cells treated with depsipeptide. A, expression of COL2A1 mRNA in SW1353 and OUMS-27 cells treated with depsipeptide for 24 h was examined by TaqMan PCR analysis. The mRNA expression levels, calculated by dividing the concentrations of human COL2A1 by those of glyceraldehyde-3-phosphate dehydrogenase and relative to those in the untreated control cells, are indicated. B, RCS cells were transiently transfected with each reporter plasmid. Depsipeptide treatment was started 24 h after the transfection and continued for 24 h. Then, cells were lysed and luciferase activity in the lysates was measured with a luminometer. C, the schema shows the part of the human COL2A1 gene structure and the fragments amplified with the indicated primers (arrows). Numbers correspond to the transcription start site of human the COL2A1 gene. D, representative data of chromatin immunoprecipitation assay. Soluble chromatin preparations from SW1353 cells treated with or without 1.85 nmol/L depsipeptide for 24 h were immunoprecipitated with the antibody against acetylated histone H3 (Ac-H3) or normal rabbit immunoglobulin G (IgG) and subjected to PCR. Aliquots of the chromatin before immunoprecipitation (Input) were also analyzed. No fragments were detected in any control samples immunoprecipitated with normal rabbit immunoglobulin G.

Fig. 3. Dose-dependent effects of depsipeptide on the expressions of extracellular matrix components mRNA in chondrosarcoma cells. SW1353 and OUMS-27 cells were treated with various concentrations of depsipeptide for 24 h. Total RNA was extracted from the cells and aggrecan, COL11A2, COL10A1, and COL1A1 mRNA levels were determined by TaqMan PCR. A, Aggrecan. B, COL11A2. C, COL10A1. D, COL1A1.
were not affected with regard to COL1A1 expression, although the untreated cells showed a down-regulation of COL1A1 from 7 to 21 days (Fig. 4E, SW1353). Depsipeptide also induced the mRNA expressions of COL2A1, aggrecan, COL11A2, and COL10A1 in OUMS cells. However, the down-regulation following the up-regulation of COL2A1, aggrecan, or COL11A2 mRNA, which was observed in SW1353 cells, was not shown in OUMS cells treated with depsipeptide (Fig. 4A-D, OUMS-27). These data suggested that long-term treatment with a relatively low dose of depsipeptide might induce, at least in part, differentiation into a hypertrophic state in chondrosarcoma cells.

In vivo antitumor effects of depsipeptide on chondrosarcoma. The antitumor activity of depsipeptide was examined in s.c. tumors of RCS developed in nude mice. Intraperitoneal administration of depsipeptide led to a significant inhibition of tumor growth in a dose-dependent manner (Fig. 5A). There were no measurable toxicities observed in the drug-treated groups (data not shown). To examine the effect of depsipeptide on the differentiation of chondrosarcoma in vivo, histologic analyses of the resected tumors were done. Although remarkable morphologic changes were not observed with H&E staining (Fig. 5B, H&E), safranin O staining showed that treatment with 10 mg/kg depsipeptide moderately down-regulated the synthesis of glycosaminoglycans (Fig. 5B, Safranin O). The accumulation of alkaline phosphatase activity is associated with hypertrophic chondrocytes (30). Depsipeptide treatment resulted in an elevation of alkaline phosphatase activity. Most of the cells strongly positive for alkaline phosphatase staining were large and round in shape, suggesting that the cells might be induced to differentiate into the hypertrophic phenotype by the intraperitoneal treatment with depsipeptide (Fig. 5B, ALP). Consistently, Alizarin red staining showed that the section of the tumor treated with depsipeptide was focally mineralized, whereas the untreated tumor was not stained with Alizarin red (Fig. 5B, Alizarin red). Furthermore, immunohistochemical staining showed that COL10, as an indicator of the hypertrophic phenotype, was significantly induced on protein level in the tumor treated with depsipeptide (Fig. 5C). These results suggested that depsipeptide might effectively inhibit tumor growth through the induction of differentiation into the hypertrophic and mineralized state in chondrosarcoma cells in vivo.

**Discussion**

Growth inhibitory activity of HDAC inhibitors has been shown for a large range of transformed cell types including solid tumors and hematologic cancers. The cell viability assay showed that IC50 values of depsipeptide for chondrosarcoma cells seemed to be similar to those for Ewing’s family tumor.

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**Fig. 4.** Differentiation of chondrosarcoma cells induced by treatment with depsipeptide. The cells were cultured in the presence or absence of 1.85 nmol/L depsipeptide and the medium was changed every 24 h. Experimental Days, each time point after the start of depsipeptide treatment, when RNA extraction was done. One microgram of total RNA was subjected to reverse-transcription reaction and quantitative real-time PCR. mRNA expression levels of matrix genes, which were relative to that in the untreated cells on day 0. A, COL2A1; B, Aggrecan; C, COL11A2; D, COL10A1; E, COL1A1 DP- depsipeptide.
cells, which we examined in a previous study, or for others such as lung, gastric, breast, and colon tumor cell lines (Fig. 1A; refs. 13, 31). As shown in other neoplasms, depsipeptide induced p21Waf1/Cip1 expression, cell cycle arrest, and apoptosis in chondrosarcoma cells (Fig. 1). It has been reported that p21Waf1/Cip1 expression related to the differentiation of chondrosarcoma is exclusively found in well-differentiated chondrosarcoma cells (32), and that the up-regulation of p21Waf1/Cip1 protein is required for the progression of chondrogenic differentiation (33). Our data suggested the possible efficacy of HDAC inhibitors on chondrosarcoma cells and encouraged us to proceed with further investigations.

During chondrogenic differentiation, the selective and combinational expression of extracellular matrix genes signifies the phenotype of differentiated cells. Among these genes, it is the mechanisms of COL2A1 transcription that have been most fully elucidated, including those in the regulation of histone acetylation and/or deacetylation. Furumatsu et al. (10) reported that trichostatin A treatments increased the COL2A1 expression as well as the acetylation levels of histone H3/H4 around the COL2A1 enhancer region containing the Sox9 binding site in human primary chondrocytes. On the other hand, our data showed that HDAC inhibitors might directly induced COL2A1 mRNA expression via the accumulation of histone acetylation in the promoter and enhancer regions in chondrosarcoma cells (Fig. 2). COL2A1, aggrecan, and COL11A2 are all regulated by the sequence-specific transcription factor Sox9 (34). Our results also suggest that there could be common mechanisms for the regulation of histone acetylation and/or deacetylation during the transcription of these three genes. Thus, the existence of Sox9 protein in the cells could perhaps play an important role in the transcriptional activation caused by the treatment with HDAC inhibitors, at least in part.

The mechanism of how differentiation is induced by HDAC inhibitors in chondrosarcoma cells has yet to be clarified. Differentiation from mesenchymal condensation to a hypertrophic or mineralized state in chondrogenesis is a process that is mediated by the combined interaction of genetic and environmental factors, including cell-cell and cell-matrix adhesion and biomechanical signals (35). Among them, transforming growth factor-β and bone morphogenetic proteins have been reported to involve the regulation of histone acetylation and/or deacetylation in their own signaling pathways. Smads, the major intracellular signal transducers of the transforming growth factor-β/bone morphogenetic protein superfamily, have been found not only to augment the association of several transcription factors with coactivators such as p300/cyclic AMP-responsive element binding protein–binding protein (36) but also to interact with corepressors such as the HDAC/Sin3A complex (37). Further investigations are needed to elucidate these processes.

Fig. 5. The antitumor activity of depsipeptide against chondrosarcoma developed in nude mice. A, the effect of intraperitoneal administration of depsipeptide on the growth of RCS-derived tumors in athymic mice was examined as described in Materials and Methods. The statistical significance between the control group and the 10 mg/kg group was shown using a one-factor ANOVA test (* P < 0.05). B, on day 25, mice were killed and tumors were prepared for histologic analyses. Each staining was done as described in Materials and Methods. Bar, 50 μm. C, COL10 immunostaining of the same specimens in (B). Streptavidin-biotin peroxidase method with methylgreen counterstain was carried out. Bottom, higher magnification of images on top. Bar, 50 μm.
needed to delineate those factors of which the activities are enhanced or inhibited by HDAC inhibitors, resulting in the induction of differentiation in chondrosarcoma cells.

It is noteworthy that although the treatment with high doses of HDAC inhibitors for 24 h inhibited COL1A1 mRNA expression in chondrosarcoma cells, the down-regulation of COL1A1 was not observed in the long-term treatment despite our expectation (Fig. 3 versus Fig. 4). It has been reported that a HDAC inhibitor, phenylbutyrate, decreases COL1A1 expression within 24 h in human lung fibroblasts by mechanisms that include cyclic AMP production and histone acetylation (38). On the other hand, COL1A1 expression has been found to be suppressed by peroxisome proliferator-activated receptor γ via its ability to inhibit p300 (histone acetyltransferase)–facilitated binding of neurofibromatosis type 1 to DNA of the promoter (39). Although the mechanism of the transcriptional control of COL1A1 gene during chondrocytic differentiation has remained unknown, we speculate that the down-regulation of COL1A1 expression during chondrocytic differentiation might be mediated by the activity of HDAC enzymes. It may explain the result indicating that the effect of HDAC inhibitors on differentiation in chondrosarcoma cells could be partial.

Recent advances in the understanding of chondrosarcoma development have suggested several molecular targets for novel adjuvant therapies for the tumor, such as the inhibition of parathyroid hormone-related protein signaling, matrix metalloproteins (3), p38 mitogen-activated protein kinase pathways (40), or the treatment with peroxisome proliferator-activated receptor-γ ligands (41). However, there have been only a few in vivo experiments using the xenograft chondrosarcoma model. In the present study, desipramine clearly indicated antitumor effects against chondrosarcoma in vivo.

In conclusion, we showed the effectiveness of HDAC inhibitors in the therapeutic application for chondrosarcomas. Accordingly, HDAC inhibitors might be a promising novel tool for use as a differentiating chemotherapy against chondrosarcomas. This is the first report showing the potential clinical usefulness of HDAC inhibitors in the treatment for chondrosarcomas.

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