ANTITUMOR EFFECTS OF HISTONE DEACETYLASE INHIBITORS ON EWING’S SARCOMA

+Sakimura, R; *Tanaka, K; *Matsumoto, Y; *Nakatani, F; *Matsunobu, T; *Matsuda, S; *Iwamoto, Y
+Dept. of Orthop. Surg. Graduate school of medical sciences, Kyushu Univ., 3-1-1 Maidashi, Higashi-ku, Fukuoka,812-8582, Japan., +81-92-642-5487, Fax: +81-92-642-5507, riku@ortho.med.kyushu-u.ac.jp

Introduction
In the majority of Ewing family of tumors (ET) including Ewing’s sarcoma and PNET, a chromosomal translocation t (11; 22) results in expression of a fusion protein EWS-Fli1. Several reports have demonstrated that EWS-Fli1 chimeric gene has the biochemical characteristics of an aberrant transcription factor, and that EWS-Fli1 is associated with tumorigenesity of ET. We previously reported that inhibition of transcription of EWS-Fli1 by treatment with antisense oligonucleotides resulted in growth arrest of various ET cell lines in vitro and in vivo. The growth arrest was observed in the G1 phase of cell cycle progression. Although little expression of p21waf1, cyclin-dependent kinase inhibitor, was observed in untreated ET cells, the antisense treatment induced expression of p21 both at mRNA and protein levels in ET cells. Moreover we have revealed that EWS-Fli1 downregulates p21 promoter activity via direct binding to the promoter. Thus, p21 could be targetted as molecular-based therapy for ET. Recent reports have demonstrated the growth inhibitory effects of histone deacetylase inhibitors (HDACI) which up-regulate p21 expression on several cancer cells. In the present study, we investigated the effect of HDACI on ET.

Materials and methods
Human ET cell lines SK-N-MC and PNKT1, and mouse fibroblast Balb3T3 were cultured in DMEM supplemented with 10%FBS. A HDACI, FK228, was generous gift from Fujisawa Pharmaceutical Co. (Osaka). Another HDACI, sodium butylate (SB), was obtained from Wako. FK228 was dissolved in 100% ethanol at a concentration of 1mg/ml and was stored at -20℃. Cells were plated at a density of 4x10^4 cells/2ml media in triplicated 35mm-dishes. Various concentration of SB or FK228 were added to cells 48h after cell seeding. Cells were trypsinized and the cell number was counted on a coulter cell counter every 24h for 4 days. Total RNA was isolated from cells treated with SB or FK228 using RNA-easy kit (Qiagen) and 1µg of total RNA were subjected to the reverse-transcription reaction and PCR for p21 mRNA expression. Cell lysates from treated cells were prepared and subjected to Western blot analysis of p21, phospho-Rb, and total Rb expression. SK-N-MC cells were subcutaneously inoculated into athymic mice (1x10^7 viable cells/mouse). Seven days after tumor inoculation, FK228 suspended in PBS was administered intraperitoneally at day-1, -5 and -9. Six mice were used in each group. Thirteen days after tumor implantation, the length(A mm) and width(B mm) of each tumor were measured and the tumor weight was calculated by the following formula:

\[ \text{Tumor weight (mg)} = \frac{1}{2} \times A \times B^2 \]

Results
We first investigated the effect of SB on the expression of p21 in SK-N-MC cells. RT-PCR and Western blot showed that both RNA and protein levels of p21 were elevated gradually in a dose-dependent manner. The growth inhibitory effect of SB on SK-N-MC cells was dose-dependent in the range from 0.625mM to 5.0mM of concentrations. The expression of p21 was also dose-dependently induced by the treatment of FK228 both at mRNA and protein levels. Accompanied by p21 induction, the expression of phosphorylated-Rb was reduced (Fig.1). Inhibition of the proliferation of ET cells, SK-N-MC and PNKT1, by FK228 was very potent. IC50 values were 0.17 and 0.12ng/ml, respectively. FK228 had weak cytotoxic activities against mouse normal fibroblast Balb3T3, for which IC50 value was 2.19ng/ml. Effects of FK228 on tumor in nude mice were next examined. As shown in Fig.2, FK228 significantly inhibited the tumor growth in a dose-dependent manner (P<0.05).

Discussion
The cell cycle inhibitor protein p21 modulates cyclin-dependent kinase activity and subsequently reduces phosphorylated-Rb, resulting in cell growth arrest. EWS-Fli1 fusion protein inhibits p21, which causes an excess of cell cycle progression. In this study, we demonstrated that HDACI induced p21 expression and inhibited proliferation of ET cells in vitro and in vivo. Phosphorylated-Rb was reduced by FK228 treatment, which might result in cell cycle arrest. FK228 showed selective cytotoxic activities against ET cells. These results suggest that HDACI might be useful as a novel target-based therapy for ET.