Combination Therapy of Histone Deacetylase Inhibitor and DNA Demethylation Agent is Effective Suppressed the Growth of Synovial Sarcoma Cells

INTRODUCTION:
HDAC inhibitors (HDACI) play multifunctional roles in a carcinogenesis, such as upregulating tumor suppressor gene expression, reducing tumor growth, inhibiting angiogenesis and inducing apoptosis and cellular differentiation. We reported that HDACI, valproic acid (VPA) inhibited the growth of synovial sarcoma.

The anti-tumor effects of DNA methyltransferase (DNMT) inhibitor against synovial sarcoma cell lines were analyzed with demethylation and re-expression of RASSF1A. RASSF1A is a tumor suppressor gene, which is not expressed in SYO-1 cell and it’s promoter region is methylated. Zebularine (Zeb) has been recently discovered mechanism-based inhibitor of DNA methylation, which is very stable and low toxicity compared with other DNMT inhibitor, such as 5-Aza-CR and 5-Aza-CdR. The ability to administer Zeb with other epigenetic therapeutics with at least additive effect has also been established. The role of epigenetic regulation of tumor progression is not clear yet.

In this study, to clarify the HDACI, VPA and DNMT inhibitor, Zeb leads to additive effects in vitro and in vivo on synovial sarcoma cell.

MATERIALS AND METHODS:
In vitro experiments
Cell culture and reagents: We used a synovial sarcoma (SYO-1) cell line. VPA was kindly supplied by Kyowa Hakko Kogyo Co., LTD. Zebularine was obtained from Wako Pure Chemical Industries, Ltd.
Detection of proliferation: SYO-1 cells were seeded in 96-well plates, these cell lines were cultured with the indicated doses of VPA (0.1, 1, 5 and 10 mM) and Zeb (0, 20, 50, 100, 200 and 400µM). After cultured with VPA and Zeb for 96 hours, a growth inhibition was evaluated using WST-1 assay (Roche), and cytotoxicity was detected using LDH assay (Roche).
Methylation specific PCR (MSP) and RT-PCR of RASSF1A: DNA extraction, MSP and RT-PCR were performed as described previously.
Western blot analysis: Total protein was extracted from cultured cells with 2mM VPA for 24 hours. Histone acetylation was detected to acetyl-histone H3 (Cell signaling). The reactive band was analyzed using Image J software. Signals were standardized to β-actin (Sigma).
In vivo experiments
Tumor growth and treatment in nude mice: 1x10⁷ of SYO-1 cells were injected into the left flank of 5-week-old BALB/c nu/nu mice. Twenty mice were divided to five groups: 1) combination of VPA gavage (80µg) by daily with VPA in drinking water and Zeb (175mg/kg), 2) combination of VPA gavage with VPA in drinking water and Zeb (80mg/kg), 3) VPA gavage only, 4) Zeb only and 5) oral administration of vehicle only. Zeb was given by repeated intraperitoneal injection in Group of 1, 2, and 4. (Table 1).

Tumor volumes were calculated, and the body weight of mice was measured every three days. These mice were sacrificed on Day 19 of the treatment by CO₂ overdose.

Table 1. Treatment group (n=4 each)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>VPA</th>
<th>Zeb</th>
<th>Mean increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VPA+Zeb</td>
<td>80µg</td>
<td>175mg/kg</td>
<td>64% VPA</td>
</tr>
<tr>
<td>2</td>
<td>VPA+Zeb1D</td>
<td>80µg</td>
<td>175mg/kg</td>
<td>64% VPA</td>
</tr>
<tr>
<td>3</td>
<td>VPA</td>
<td>30µg</td>
<td>no treatment</td>
<td>64% VPA</td>
</tr>
<tr>
<td>4</td>
<td>Zeb</td>
<td>no treatment</td>
<td>175mg/kg</td>
<td>no treatment</td>
</tr>
<tr>
<td>5</td>
<td>Vehicle</td>
<td>no treatment</td>
<td>no treatment</td>
<td>no treatment</td>
</tr>
</tbody>
</table>

Statistical analysis: Statistical analysis was performed using Microsoft Excel 2003. Significance was calculated using the t-test for non-paired samples.

RESULTS:
In vitro: Anti-proliferative effect of VPA and Zeb in synovial sarcoma cell
In vitro treatment with both VPA and Zeb strongly suppressed the growth of SYO-1 cells, and the anti-proliferative effect was dose-dependent. Combination of VPA and Zeb also induced cytotoxic effect a cell proliferation compared with single agents in SYO-1. (Fig 1).

Figure 1. Effect of VPA and zebularine in synovial sarcoma cell

MSP and RT-PCR for SYO-1 revealed that RASSF1A promoter was methylated and RASSF1A mRNA was not expressed. After the treatment with Zeb, de-methylation of RASSF1A promoter and re-expression of the RASSF1A mRNA were confirmed. Histone H3 acetylation was increased in VPA, Zeb and VPA + Zeb treatment group, the ratio was 1.53, 1.00 and 1.10, respectively compared with Control. (Fig 2)

Figure 2. DNA Methylation and RNA re-expression of RASSF1A gene

DISCUSSION:
In this study, VPA increases histone acetylation and Zeb induced DNA demethylation, combination of these agents has additive effect of epigenetic gene regulation that inhibited the cell growth in vitro and in vivo. Further studies needed to clarify the epigenetic gene regulation in vitro. The combination therapy of VPA and Zeb also strongly suppressed the tumor growth on nude mice. However, the weight loss of mice was detected after this combination therapy. It is necessary to examine appropriate dose for this combination therapy. Combination of the HDACI and DNMT drug might be a new treatment for synovial sarcoma through adjusting epigenetic of DNA.