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Restoration of TGFβ signaling in human prostate cancer by histone deacetylase inhibitor MS-275

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14. ABSTRACT: The goal of the current grant is to investigate the potential antitumor activity of histone deacetylase inhibitor MS-275 along with the activation of TGFβ signaling pathway with the restoration of TGFβ receptor II. As presented in our initial proposal, prostate cancer cell line LNCaP has reduced expression in TGFβRII, which is due to the promoter histone deacetylation. Subsequent treatment with chromatin remodeling agent MS-275 was able to restore the expression of TGFβRII. We hypothesized that the restoration of TGFβ signaling may contribute to the antitumor activity of MS-275. In the past a year and half, we have focused our effort to identify the re-expression of TGFβRII in vivo, and investigate the antitumor activity of MS-275 in several relevant prostate cancer model. We observed dose and time dependent upregulation of TGFβ1 and TGFβRII in LNCaP cells. A cDNA microarray of LNCaP cells (untreated vs. MS-275 treated) has been done, and the data is being analyzed. We observed the antitumor activity of MS-275 in two different models of prostate cancer xenografts. Immunohistochemistry analysis of reactivation of TGFβ1 in vivo is on going.

15. SUBJECT TERMS
prostate cancer, Histone deacetylase inhibitors, TGFβ signaling

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Introduction:

The goal of the current grant is to investigate the potential antitumor activity of histone deacetylase inhibitor MS-275 along with the activation of TGFβ signaling pathway with the restoration of TGFβ receptor II. As presented in our initial proposal, prostate cancer cell line LNCaP has reduced expression in TGFβRII, which is due to the promoter histone deacetylation. Subsequent treatment with chromatin remodeling agent MS-275 was able to restore the expression of TGFβRII. We hypothesized that the restoration of TGFβ signaling may contribute to the antitumor activity of MS-275. In the first one and half year of study, we had focused our effort to identify the re-expression of TGFβRII in vivo, and investigate the antitumor activity of MS-275 in several relevant prostate cancer model. Since the last progress report, we have focused our effort to demonstrate a) the epigenetic mechanism of TGFβRII expression by HDAC inhibitors, b) the effect of HDAC inhibitors on androgen receptor signaling.

Body:

Specific Aim 1): To determine whether loss of TGFβRII expression in a human prostate carcinoma cell line is due to transcriptional epigenetic repression.

To demonstrate the loss of expression of TGFβRII in LNCaP cells, we performed chromatin immunoprecipitation assay (CHIP). As shown in Figure 1C, the promoter region of TGFβRII and its primary ligand TGFβ1 were un-acetylated in control (drug free) condition. Addition of MS-275 at 1 and 2 μM increased the promoter acetylation, which corresponds to the upregulation of the gene expression (as demonstrated in figure 1A 1B). The current CHIP assay (figure 1C) complements with the previous RT-PCR results (figure 1a 1b), establish the epigenetic silencing nature of these two genes in prostate cancer LNCaP cells.

Specific Aim 2): To determine whether re-expression of TGFβRII by HDACI is associated with the restoration of TGFβ signaling and contributes to HDACI antitumor activity.

We also examined the TGFβRII expression levels in other prostate cancer cell lines – PC3, LAPC4, and DU145. All these cell lines exhibited robust expression level of TGFβRII and its ligands. HDAC inhibitors could not further upregulate the expression. In addition, the Smad pathway components that downstream of TGFβRII signaling in all these cell lines were not altered by the HDAC inhibition. Therefore, we concluded that the upregulation of TGFβRII signaling by HDAC inhibitors were limited to the LNCaP cells. In vitro, cell proliferation assays confirmed statistically significant inhibition over single agents when HDAC inhibitors were combined with exogenous TGFβ1 (figure 2). However, due to the fact that HDAC inhibitors also target many growth pathways that are independent of TGFβRII signaling, there were significant levels of inhibition on the growth of cancer cells both in vitro and in vivo regardless of the TGFβRII status (table 1, figure 3, 4 and 6). The conclusion, therefore, is that HDAC inhibitors may upregulate TGFβRII that partially mediates the antitumor activity of HDAC inhibitors.

Specific Aim 3): To evaluate the antitumor effect of HDACIs in combination with anti-androgen treatment (androgen ablation).

Our initial hypothesis is that HDAC inhibitors may synergies with androgen ablation therapy. However during our in vitro experiments and clinical trials of HDAC inhibitors in prostate cancer patients, we observed that HDAC inhibitor’s antitumor activity does not complement with the reduction of androgen ablation. In vitro, HDAC inhibitors may upregulate PSA expression, which is a target gene of androgen signaling pathway under total growth factor and androgen starvation condition (table 2), but not in full serum condition (figure 5). The possible modulation of PSA by HDAC inhibitors has not been fully investigated. Under our experimental conditions, we did not observed PSA induction in hormone sensitive cell lines at the gene expression level, but
we observed increased protein levels in LAPC4 treated cells. These results suggest a possible PSA modulation by MS-275 at the posttranscriptional level, or perhaps by increasing its secretion. Further studies will be necessary to elucidate this observation. The induction of PSA protein level in association with inhibition of cell proliferation following in vitro MS-275 treatment suggest that changes in PSA levels should not be considered a primary endpoint in clinical trials involving HDAC inhibitors. However, PSA induction may represent a readable biological effect of HDAC inhibition and a potential biomarker for drug exposure.

Key Research Accomplishment
1. MS-275 upregulated both TGFb1 and TGFbRII expression in LNCaP cells
2. MS-275 was effective in vivo targeting LNCaP growth
3. The growth inhibition of MS-275 was cytostatic with G1 arrest.
4. MS-275 was also effective against prostate cancer cell growth in the bone environment
5. Established the epigenetic nature of TGFBRII and TGFb1 silencing in LNCaP cells.
6. Observed the PSA induction by MS-275 that is in contrast with the antitumor activity of the drug.

Reportable Outcome
A manuscript reporting the in vivo antitumor activity of MS-275 in various pre-clinical prostate cancer models has been submitted it to the journal of prostate.

Conclusion
MS-275 was effective against prostate cancer growth in vitro and in vivo. The re-expression of TGFBRII and its ligand TGFb1 in LNCaP cells may be mediators for the MS-275 antitumor activity. However, the upregulation of TGFb1 may be able to promote the tumor microenvironment facilitating tumor growth.

Although a clinical role of HDAC inhibitors is already a reality, their general clinical utility will likely depend greatly on the future development of molecular or cellular predictors of their antitumor activity. Molecular targeted combination therapies of post-transcription modifiers such HDAC inhibitors with standard therapies (chemotherapy and radiation therapy) and novel biologics including angiogenesis inhibitor represent an exciting challenge. Understanding the nature of the molecular basis of the selectivity of HDACs and designing specific HDACs inhibitors will be critical to further exploit the potential of this novel class of agents in the treatment of solid tumors including prostate cancer.
Figure 1A. Dose-dependent gene upregulation of TGFβ1 and TGFβRII in LNCaP cells after MS-275 treatment.

Figure 1B. Time-dependent gene upregulation of TGFβ1 and TGFβRII in LNCaP cells after MS-275 treatment.

Figure 2. Cell proliferation assay of LNCaP with solvent control, MS-275, TGFβ1 only, and as combination. * P<0.05 vs. single agent student t test.
Chromatin Immunoprecipitation PCR (CHIP)

LNCaP cells were treated with 1 or 2 μM of MS-275 for 24 hours, cells were subject to CHIP assay using Acetyl-H4 antibody (Upstate) based on the protocol included in the CHIP assay kit (Upstate), and DNA fragments containing acetyl-H4 were amplified using PCR primers specific for TGFβ1 and TGFβRII promoters. Total DNA without acetyl-H4 immunoprecipitation was used as positive input control.
Figure 3. A) PC-3, DU-145 and LNCaP cells were treated with 0, 0.1, 0.5, 1.0 and 2.0 μM of MS-275 for 48 hours. The viable cell numbers at Day 2 were measured, and the viable cells numbers at day 0 were normalized to 100%. B) Similar experiments were done as in A) using 0.5 μM of MS-275. Then cells were harvested and stained with Annexin V and PI, and analyzed with FACS.

Table 1. Cell cycle analysis of three prostate carcinoma cell lines with and without MS-275 treatment (0.5 μM for 48 hours). The cell population at each specific stage was expressed as % (control -> treated).

<table>
<thead>
<tr>
<th></th>
<th>SubG0 (%)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU-145</td>
<td>5 -&gt; 75</td>
<td>55 -&gt; 13</td>
<td>19 -&gt; 9</td>
<td>21 -&gt; 3</td>
</tr>
<tr>
<td>LNCaP</td>
<td>1 -&gt; 4</td>
<td>62 -&gt; 73</td>
<td>17 -&gt; 5</td>
<td>20 -&gt; 22</td>
</tr>
<tr>
<td>PC-3</td>
<td>7 -&gt; 10</td>
<td>44 -&gt; 52</td>
<td>24 -&gt; 10</td>
<td>25 -&gt; 28</td>
</tr>
</tbody>
</table>
Figure 4. Approximately 2 million LNCaP cells were injected into male nude mice s.c. When the tumors were palpable, daily MS-275 at 20 mg/kg/day were administrated for 5 weeks. Tumor volumes were followed with caliper measurements. Data were mean and SEM, * P < 0.05, student t test.

<table>
<thead>
<tr>
<th>MS-275 (μM)</th>
<th>LNCaP</th>
<th>LAPC4</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. PSA gene expression in LNCaP and LAPC4 cells were analyzed by RT-PCR with and without MS-275. PC-3 cell line that does not express PSA was used as a negative control.
Figure 6. (A) Tumor burden in the control and MS-275 treated groups were followed by in vivo imaging of luciferase activity. The values for each group was expressed as mean and standard error, *, P < 0.05. (B) Representative luciferase imaging of one mouse from each group at day 1, 22 and 36 post-injection. (C) Representative radiography of tumor bearing legs in control and MS-275 treated mice at the end of the treatment. (D) The body weight and tumor weight of the mice at the end of the experiment expressed as % of the untreated control group with standard deviation. The net tumor weights were obtained by subtracting the normal contralateral tibia from the tumor-bearing tibia. *, P < 0.05. (E) Representative H&E at 10X and 40X magnification. a = bone matrix, b = tumor cells, c = bone marrow cells. Osteoclasts were shown as multinuclea cells indicated by arrow. (F) Western blots of tumor tissue samples showing increased histone H3 acetylation, and p21 expression in MS-275 treated mice.
PSA production was examined in early and late passages of the prostate cancer cell lines LAPC4 and PC3. Androgen-dependent cell line LAPC4 demonstrated two-fold induction of PSA after 24hr treatment with 1 μM MS-275. No PSA production was observed in androgen receptor-negative prostate cancer cell line PC3.

Table 2.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>Total PSA (ng/ml)</th>
<th>Total Protein (mg/ml)</th>
<th>Normalized PSA (mg/ml)</th>
<th>FOLD CHANGE over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAPC4 p46</td>
<td>Control</td>
<td>1.84</td>
<td>4.34</td>
<td>0.42</td>
<td>1.00</td>
</tr>
<tr>
<td>LAPC4 p46</td>
<td>1μM MS-275</td>
<td>4.02</td>
<td>4.25</td>
<td>0.95</td>
<td>2.23</td>
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<tr>
<td>LAPC4 p77</td>
<td>Control</td>
<td>3.15</td>
<td>4.32</td>
<td>0.73</td>
<td>1.00</td>
</tr>
<tr>
<td>LAPC4 p77</td>
<td>1μM MS-275</td>
<td>7.95</td>
<td>3.65</td>
<td>2.06</td>
<td>2.82</td>
</tr>
<tr>
<td>PC3</td>
<td>Control</td>
<td>&lt;0.1</td>
<td>5.05</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC3</td>
<td>1μM MS-275</td>
<td>&lt;0.1</td>
<td>4.81</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
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