Histone Deacetylase Inhibitor Romidepsin Enhances Anti-Tumor Effect of Erlotinib in Non-small Cell Lung Cancer (NSCLC) Cell Lines

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Introduction: Most epidermal growth factor receptor (EGFR) mutant non-small cell lung cancers (NSCLCs) are sensitive to EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib or gefitinib, but many EGFR wild type NSCLCs are resistant to TKIs. In this study, we examined the effects of the histone deacetylase inhibitor, romidepsin, in combination with erlotinib, in NSCLC cell lines and xenografts.

Methods: For in vitro studies, nine NSCLC cell lines with varying mutation status and histology were treated with erlotinib and romidepsin alone or in combination. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays were performed to determine the concentration that inhibits 50% (IC50) value of each drug or the combination. For in vivo studies, NCI-H1299 xenografts were inoculated subcutaneously into athymic nude mice. Romidepsin and/or erlotinib were injected intra-peritoneally after tumors developed and tumor sizes were measured.

Results: We found that romidepsin increased the sensitivity of erlotinib synergistically in all nine NSCLC cell lines including EGFR and KRAS wild type cell lines, KRAS mutant cell lines, and TKI resistant EGFR mutant cell lines. This effect was partially due to enhanced apoptosis. Furthermore, cotreatment of erlotinib and romidepsin inhibited NCI-H1299 xenograft growth in athymic nude mice.

Conclusions: These observations support a role for the combination of a histone deacetylase inhibitor and a TKI in the treatment of NSCLCs.

Key Words: Erlotinib, Romidepsin, Non-small cell lung cancer, Epidermal growth factor receptor, KRAS.

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ORIGINAL ARTICLE

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romidepsin and erlotinib on the sensitivity of EGFR wild type and mutant NSCLC cell lines resistant to TKI therapy.

**MATERIALS AND METHODS**

**Cell Lines and Drugs**

Nine NSCLC cell lines (Table 1) were cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 5% fetal bovine serum and incubated in humidified air and 5% CO2 at 37°C. The cell lines were established by us, DNA fingerprinted, and free of mycoplasma by molecular tests (iNTRON Biotechnology, Korea). Romidepsin was provided by Fujisawa Pharmaceutical Co. (Japan) (This drug is now the property of Gloucester Co., MA). Erlotinib (Tarceva) (OSI Pharmaceuticals, Inc., NY) was purchased from UT Southwestern pharmacy.

**DNA Isolation and Polymerase Chain Reaction**

DNA was isolated from NSCLC cell lines and polymerase chain reaction was performed to determine the KRAS and EGFR mutation status of each cell line according to previously reported methods.19

**MTS Assay**

Drug sensitivity was measured by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI). Two × 10^3 cells were seeded into each well of a 96-well microtiter plate. After 24 hours, the cells were treated with varying concentrations (0.01–250 μM) of erlotinib either in the absence or presence of 1 ng/ml romidepsin for 72 hours at 37°C. MTS solution was added to each well, and the plates were incubated for 1 hour at 37°C. Optical density was measured at 490 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA). Each experiment was carried out in eight replicate wells for each drug concentration and repeated twice. The concentration that inhibits 50% (IC50) value was defined as the concentration needed for a 50% reduction in the absorbance calculated based on the cell viability curves.

**Apoptosis Assay**

Apoptosis assay was performed using cell death detection enzyme-linked immunosorbent assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s protocol.

**Xenograft Animal Models**

Five × 10^6 NCI-H1299 cells were resuspended in 200 μl of RPMI 1640 and inoculated into the subcutaneous tissue of the flanks of 25-week-old female Bagg Albino (BALB/c) athymic nude mice (Charles river Laboratories, Wilmington, MA). After tumors developed, romidepsin was injected intraperitoneally into the mice three times at 4 day intervals (1.2 mg/kg body weight). Erlotinib was injected daily 5 days a week (50 mg/kg body weight). The 1 × phosphate-buffered saline solution was used as a control solution. Tumor sizes were measured and calculated from the following formula: tumor size = L × W^2/2, where L and W represent the length and the width of the tumor mass respectively.

**Statistical Analysis**

The Loewe additivity model was used to characterize drug interaction between erlotinib and romidepsin. In this model, the combination index (CI) was defined as CI = d1/D1,y1 + d2/D2,y2, where d1 and d2 are the doses of drug 1 and drug 2 in mixture, which produces an effect y, D1,y1 and D2,y2 are the doses that produce the same effect y when using alone.20,21 If the CI is equal, less than, or greater than 1, the combination dose (d1, d2) is called as additive, synergistic or antagonistic, respectively. We also used isobologram to illustrate the drug interaction geometrically.20 The Student’s t test was used to determine the potential differences between the control group and the drug treatment groups. p value <0.05 was considered statistically significant.

**TABLE 1. The IC50 Values of Romidepsin and Erlotinib in NSCLC Cell Lines**

<table>
<thead>
<tr>
<th>Histology</th>
<th>EGFR</th>
<th>KRAS</th>
<th>Romidepsin IC50 ± SD (μM)</th>
<th>Erlotinib IC50 ± SD (μM)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H1299</td>
<td>LC</td>
<td>wt</td>
<td>7.6 ± 0.2</td>
<td>78.0 ± 0.3</td>
<td>1.5</td>
</tr>
<tr>
<td>NCI-2882</td>
<td>AD</td>
<td>wt</td>
<td>8.3 ± 0.2</td>
<td>89.0 ± 0.4</td>
<td>1.8</td>
</tr>
<tr>
<td>HCC95</td>
<td>SQ</td>
<td>wt</td>
<td>8.2 ± 0.2</td>
<td>88.0 ± 0.3</td>
<td>1.9</td>
</tr>
<tr>
<td>NCI-H23</td>
<td>AD</td>
<td>wt</td>
<td>9.4 ± 0.2</td>
<td>99.0 ± 0.4</td>
<td>2.2</td>
</tr>
<tr>
<td>NCI-H157</td>
<td>SQ</td>
<td>wt</td>
<td>9.5 ± 0.2</td>
<td>100.0 ± 0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>LC</td>
<td>mut</td>
<td>10.6 ± 0.2</td>
<td>111.0 ± 0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>NCI-H1975</td>
<td>AD</td>
<td>mut</td>
<td>11.6 ± 0.2</td>
<td>122.0 ± 0.6</td>
<td>3.5</td>
</tr>
<tr>
<td>NCI-H820</td>
<td>AD</td>
<td>mut</td>
<td>13.6 ± 0.2</td>
<td>133.0 ± 0.7</td>
<td>4.0</td>
</tr>
<tr>
<td>NCI-H1650</td>
<td>AD</td>
<td>mut</td>
<td>14.6 ± 0.2</td>
<td>144.0 ± 0.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*The mutation status of NSCLC cell lines were determined as described in Materials and Methods.
* The IC50 values of erlotinib and romidepsin were defined as the concentration needed for a 50% reduction in the absorbance calculated based on the cell viability curves.
* CI combination index. Values <1 are interpreted as synergism. (see Materials and Methods).
* EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; LC, large cell; AD, adenocarcinoma; SQ, squamous carcinoma; wt, wild type; mut, mutant; IC50, concentration that inhibits 50%.
RESULTS

Romidepsin Decreases the IC₅₀ Values of Erlotinib in NSCLC Cell Lines

Nine NSCLC cell lines of varying EGFR and KRAS mutation status were chosen to study, including three cell lines containing wild type EGFR and KRAS, three containing mutant KRAS and three containing mutant EGFR but resistant to TKIs. These NSCLC cell lines also had various histologies including adenocarcinoma, squamous carcinoma and large cell types (Table 1). We first examined the cytotoxicity of romidepsin in these NSCLC cell lines. The IC₅₀ values of romidepsin ranged relatively little from 1.3 to 4.9 ng/ml (Table 1). As we selected NSCLC cell lines resistant to TKI therapy for this study, the IC₅₀ values for erlotinib (8.6–115 μM) in these cell lines were considerably higher than the upper value indicative of clinical sensitivity (2.5 μM).22

For the combined treatment, the NSCLC cell lines were treated with varying concentrations (0.01–250 μM) of erlotinib either in the absence or presence of 1 ng/ml romidepsin. MTS assays were performed to determine the cell viability and pairs of cell viability curves for each cell line are shown in Figure 1A. We found that in the presence of 1 ng/ml romidepsin, the IC₅₀ values of erlotinib in six of nine NSCLC cell lines examined decreased to less than 2.5 μM. The CI has been calculated, and <1 is considered synergism. The CI values ranged from 0.25 to 0.77 in these cell lines (Table 1) indicating that romidepsin and erlotinib showed synergism in all the NSCLC cell lines examined. A dose-normalized IC₅₀ isobologram was also made and shown in Figure 1B, illustrating the synergism geometrically.20

Combination of Romidepsin and Erlotinib Induces Apoptosis in NSCLC Cell Lines

We next examined whether inducing apoptosis played a role in the ability of romidepsin to sensitize lung cancer cells to erlotinib. NCI-H1299 and NCI-H23 were treated with either 5 μM erlotinib or 1 ng/ml romidepsin, or the combination of these two drugs. After 72 hours, apoptosis was determined using the cell death detection enzyme-linked immunosorbent assay kit (Roche Applied Science). The mono- and oligonucleosomes indicating apoptosis were detected by antibodies against DNA and histones, and quantified by densitometry. As shown in Figure 2, while 5 μM of erlotinib and 1 ng/ml of romidepsin did not induce obvious apoptosis, cotreatment of erlotinib and romidepsin caused a 2.3 (p = 0.01) and 2.85 (p = 0.002) fold increase in apoptosis in NCI-H1299 and NCI-H23 cells, respectively.

Coadministration of Romidepsin and Erlotinib Shows Inhibition on NCI-H1299 Cell Line Xenografts

We further studied the combined effect of romidepsin and erlotinib on tumor growth in vivo. We injected 5 × 10⁶ NCI-H1299 cells subcutaneously into 20 female BALB/c athymic nude mice. After visible tumors were seen at day 7, the mice were divided into four groups (five mice per group). One group was used as a phosphate-buffered saline treated control. The other three groups were intraperitoneally injected with either romidepsin alone, erlotinib alone or the combination of both agents. Tumor size was measured at the indicated days. After 20 days, the mice were killed. As shown in Figure 3, at day 20, although erlotinib and romidepsin treatment alone suppressed NCI-H1299 cell line xenograft growth to 72% (p = 0.47) and 43% (p = 0.08) respectively compared with the phosphate-buffered saline treated control group, these were not statistically significant. Only the combined treatment inhibited NCI-H1299 xenograft growth to 28%, causing a significant decrease on tumor growth (p = 0.04).

DISCUSSION

EGFR TKIs such as erlotinib and gefitinib have been found to be efficient in the treatment of NSCLCs that express mutant EGFR.3,4 However, resistance to TKIs develops in some EGFR mutant NSCLCs. Furthermore, the majority of NSCLCs containing wild type EGFR are resistant to EGFR TKIs.22 To solve this problem, several new TKIs that have a broader spectrum of kinase activities have been developed to treat NSCLCs. For example, EXEL-7647 (XL647), a novel spectrum-selective kinase inhibitor with potent activity against the EGFR and vascular endothelial growth factor receptor tyrosine kinase families, has shown efficiency in therapy for both wild type and mutant EGFR in vitro and in vivo.23 In addition, combinational therapies have been used to overcome NSCLC resistance to EGFR TKIs. For example, the combined use of erlotinib and the humanized vascular endothelial growth factor receptor monoclonal antibody bevacizumab in advanced, chemotherapy-refractory NSCLCs has shown promising results.24

Our data indicated that the HDAC inhibitor romidepsin decreased the IC₅₀ values of erlotinib in NSCLC cell lines with wild type EGFR, including KRAS mutant cell lines which are considered to be particularly resistant to EGFR TKIs. These cell lines are composed of different histologic types including adenocarcinoma, squamous carcinoma and large cell types. Since most of EGFR mutant cell lines are very sensitive to erlotinib, we examined three which exhibit erlotinib resistance: NCI-H1975 and NCI-H820 cell lines have a secondary T790M mutation, which is responsible for the resistance; NCI-H1650 cell line has a homozygous deletion of phosphatase and tensin homolog deleted on chromosome 10 and therefore no detectible phosphatase and tensin homolog deleted on chromosome 10 protein level, which is associated with resistance to TKIs in human tumors.25 We found that addition of romidepsin also increased the sensitivity of erlotinib in TKI resistant EGFR mutant NSCLC cell lines. Our data indicated that the HDAC inhibitor romidepsin and TKI erlotinib showed synergy in all nine NSCLC cell lines examined.

The underlying mechanisms of this synergistic effect are not known. Besides the secondary T790M mutation, several other mechanisms have been proposed to explain the resistance of NSCLCs to EGFR TKIs. It is reported that epithelial to mesenchymal transition is a determinant of sensitivity of NSCLCs to EGFR inhibition.26 Epithelial to mesenchymal transition is characterized by the combined loss of epithelial cell junction proteins such as E-cadherin and the
FIGURE 1. Romidepsin synergistically increased the sensitivity of erlotinib in non-small cell lung cancer (NSCLC) cell lines. A, Nine NSCLC cell lines were treated with varying concentrations of erlotinib either in the absence or presence of romidepsin (1 ng/ml) for 72 hours. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and representative pairs of cell viability curves are shown. B, The dose-normalized isobologram for erlotinib and romidepsin with normalization of dose with IC_{50} to unity on both x and y axis. All the points represent the drug combination that yield 50% cell reduction. If the point falls on the lower left part of the graph, synergism is indicated.
106 NCI-H1299 cells were injected subcutaneously into each grafts by erlotinib and romidepsin coadministration. Five × 10⁶ NCI-H1299 cells were injected subcutaneously into each of twenty female BALB/c athymic nude mice. These mice were divided into four groups at day 7 after tumor development. They were injected with either 1 × phosphate-buffered saline (PBS), romidepsin alone, erlotinib alone or the combination. Romidepsin was administered 3 times at 4-day intervals (1.2 mg/kg body weight). Erlotinib was administered 5 days a week (50 mg/kg body weight). Tumor sizes were measured at the indicated days. Results are shown as means ± SEM of groups of 5 mice. Statistical significance was determined by Student’s t test (*p < 0.05 versus control).

Gain of mesenchymal markers such as vimentin or fibronectin.27 Restoring E-cadherin expression has been shown to increase sensitivity to EGFR inhibitors in NSCLCs.13 Persistent activity of MAPK or/and AKT pathway has also been related to EGFR TKI resistance. Treatment of specific inhibitors of MAPK or AKT pathway can increase the sensitivity of NSCLC cell lines to TKIs. Romidepsin can regulate expression of many genes. It was reported that romidepsin treatment led to altered expression of cyclin A, cyclin E, and p21, reduced protein levels of ErbB1, ErbB2, and Raf-1.28 Furthermore, romidepsin inhibited ERK1/2 MAPK activities.28 Modification of important players in signal transduction pathways, or other important cellular events by romidepsin may account for the synergy.

In our studies, in the presence of romidepsin at the concentration of 1 ng/ml, the IC₅₀ values of erlotinib in six out of nine NSCLC cell lines decreased to <2.5 μM, which is considered to be sensitive to erlotinib in vitro,22 and approximately corresponds to the plasma steady-state concentration of erlotinib in patients treated with a dose of 150 mg daily.29 The concentration of romidepsin used for our in vitro combination treatment is far below 25 ng/ml that corresponds to 50% of the free drug concentration in plasma from lung cancer patients receiving this drug at the maximum tolerated dose.28 This suggested that the combined treatment of romidepsin and erlotinib will be beneficial in clinical trials. A recent study indicated that although romidepsin exhibited modest clinical efficacy in lung cancer patients at the tested dose and schedule, the biologic effects mediated by romidepsin supported the evaluation of this HDAC inhibitor in combination with other targeted agents in lung cancer patients.18 Our data indicates that combination of erlotinib with romidepsin may benefit NSCLC patients not predicted to respond to TKI therapy.

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REFERENCES


