Anticancer Effect of a Novel Histone Deacetylase Inhibitor in Cell Model

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ABSTRACT

Histone deacetylases (HDACs) are potential therapeutic targets for the treatment of hematologic and solid tumor malignancies due to their overexpression and/or increased activity in various cancers. Although HDAC inhibitors exhibit significant inhibition ability in hematological cancers, their effect in solid tumors has not been satisfactory. Here, we propose a novel HDAC inhibitor, N-hydroxy-4-((1R,2R)-2-phenylcyclopropanecarboxamido)benzamide (HPCPCB), as a chemotherapeutic drug for solid tumors. First, we examined the cytotoxicity of HPCPCB in various lung and breast cancer cells and their corresponding normal cells using MTT assay. HPCPCB showed significant growth inhibition on various cancer cells, while there was no serious cytotoxicity against normal cells. Results of flow cytometry showed that HPCPCB caused cell cycle arrest at G1 phase. Western blotting indicated that proteins required for cell cycle progression such as CDC25A, CDK4 and cyclin E were reduced and the cell cycle inhibitor p21 was up-regulated by HPCPCB treatment. Furthermore, HPCPCB inhibited HDAC activity and induced an increase in acetylated histone proteins H3 and H4 and non-histone proteins p53 and tubulin. Our findings suggest that HPCPCB is a potent HDAC inhibitor and has potential in cancer treatment.

Key words: HDAC, acetylation, cancer, cell cycle arrest

Introduction

Chemotherapy is one of the standard therapies for cancer treatment and has an important role as strategic and palliative treatment (Danesi et al., 2003; Mariette et al., 2007). However, limited response, severe adverse side effect, and poor prognosis make the clinical use of chemotherapy unsatisfactory. Therefore, it is important to develop novel anticancer drugs with more curative effect and less side effects for cancer treatment. A few target agents, such as bevacizumab and erlotinib, have been used with regular chemotherapy drugs in combinative treatment to raise the benefit and minimize the side effect (Sandler et al., 2006; Shepherd et al., 2005). Therefore, it is an imperative task to develop novel molecular targeted drugs.

Epigenetic changes are clearly associated with tumorigenesis (Bhalla, 2005). Recent studies showed that the modification patterns of histones influence overall survival and can be used as prognosis prediction biomarkers (Barlesi et al., 2007; Manuyakorn et al.). Histone deacetylases (HDACs) are histone modification enzymes, which remove the acetyl-groups from histones and regulate chromatin structure and gene expression epigenetically. In addition to histones, HDACs have many other substrates which are involved in the regulation of cellular function, such as p53, HSP90, tubulin, and of various transcription factors (Xu et al., 2007). The acetylation status of non-histone proteins have influence on its stability (microtubulin, p53, and SMAD7), transcriptional activity (p53, E2F family, NFXB, MEF2, and...
TF2B), protein-protein interaction (STAT3, Retinoblastoma, and β-catenin), and protein-DNA binding (p53, E2F family, NFκB) (Minucci and Pelicci, 2006). Overexpression and/or increased activity of HDACs in various cancers and low basal level in normal cells make HDACs potential therapeutic targets for cancer treatment (Ellis et al., 2009; Minucci and Pelicci, 2006; Witt et al., 2009). The potent antitumor activities of HDAC inhibitors have been demonstrated in cutaneous T-cell lymphoma (Mann et al., 2007). HDAC inhibitors show antitumor effect through inducing cell cycle arrest, differentiation and apoptosis in various cancer cells (Bolden et al., 2006; Xu et al., 2007). Therefore, development of HDAC inhibitors is a feasible scheme for novel anticancer drugs used in solid tumors.

In this study, we propose a novel HDAC inhibitor, N-hydroxy-4-((1R,2R)-2-phenylcyclopropanecarboxamido)benzamide (HPCPCB), a derivative of USA Food and Drug Administration approved HDAC inhibitor vorinostat (SAHA, suberoylanilide hydroxamic acid), as potential anticancer drug for treating solid tumors. The antitumor ability and mechanism of actions of HPCPCB were studied in lung and breast cancer cell models. We found that HPCPCB induced cell cycle arrest and potently inhibited the activity of HDAC resulting in the acetylation of histone and non-histone proteins. Our data suggest that HPCPCB is an HDAC inhibitor and have potential to be applied as targeted anticancer drug.

Materials and Methods

HPCPCB

The compound N-hydroxy-4-((1R,2R)-2-phenylcyclopropanecarboxamido)benzamide, namely HPCPCB, was synthesized by one of the co-authors, Dr. Chein-Tien Chen. HPCPCB was obtained in powdery form and was dissolved in 100% DMSO for further dilution in cell culture medium.

Cell lines

The normal human lung cell line IMR90, the human lung adenocarcinoma cell lines A549 and H1299, and the large cell lung cancer cell line H460 were purchased from American Tissue Culture Company (Rockville, Maryland, USA). Normal human breast cell MCF-10A and breast cancer cell MDA-MB-231 were kindly provided by Dr. Ying-Jan Wang (Department of Environmental and Occupational Health, National Cheng Kung University).

Cytotoxicity/MTT assay

Cells were seeded at 1×10^5 cells/well in 6-well plates and maintained in culture medium containing 10% Fetal Bovine Serum (FBS, Gibco, Invitrogen, Carlsbad, CA) for 16 hours. Different concentrations of HPCPCB or solvent control DMSO were added into each plate and incubated for 48 hours. After treating with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C, cell viability was determined by measuring the absorbance at 570 nm wavelength.

Cell cycle analysis

The assay was performed according to Kuo et al. (Kuo et al., 2004). A549 was pretreated with nocodazole (200 ng/ml) (Sigma-Aldrich) for 24 hours to arrest cells at the G2/M phase. Cells were released into G1 phase by replacing medium with fresh medium, then DMSO or 7 μM HPCPCB was added. Cells were collected at various time points and the cell cycle distribution was analyzed by FACScan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Western blot analysis

After treating with solvent control DMSO or HPCPCB, cell lysates were collected and immunoblotted for various proteins under the conditions described as below: p21 (1:1000), CDK4 (1:1000), GAPDH (1:2000) (Santa Cruz, Santa Cruz, CA, USA); Cdc25A (1:1000, Abcam, Cambridge, England); acetyl-histone H3 (1:1000), acetyl-histone H4 (1:1000) (Upstate, Millipore, Billerica, MA, USA); acetyl-p53 (Lys382) (1:500, Cell signaling, Danvers, MA, USA); acetyl-tubulin (1:1000, Sigma-Aldrich).

Histone deacetylases (HDAC) activity assay

The HDAC activity was measured with an HDAC fluorescent activity assay kit (BIOMOL Inc, Plymouth Meeting, PA, USA) according to the manufacturer’s instructions. Briefly, the cell lysate were added to the diluted HPCPCB and SAHA, and then the substrate was added. After incubation for
10 minutes at 25°C, developer was added to the samples to stop the reaction. After incubation for 10 min, luminescence was recorded on a SpectraMax® M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Results**

**HPCPCB inhibits cell growth of various lung and breast cancer cell lines**

HPCPCB is a synthetic derivative based on clinical anticancer drug SAHA. Cytotoxicity of HPCPCB was assayed in the normal human lung cell line IMR90, and various human lung cancer cell lines (A549, H1299, and H460); breast normal cell line MCF-10A and breast cancer cell line MDA-MB-231. After treating the cell lines with different concentrations of HPCPCB for 48 hours, cell viability was measured by the MTT assay and the IC50 of each cell was calculated. As shown in Table 1, HPCPCB showed significant growth inhibition on various cancer cells with IC50 doses ranging from 6.23~6.91 μM, while there was no serious cytotoxicity against normal human lung cell IMR90 up to 20 μM.

**HPCPCB causes cell cycle G1 phase arrest**

In order to verify the cytotoxic effect of HPCPCB, we measured the cell cycle distribution after HPCPCB treatment in a synchronized cell population. After pretreatment with nocodazole for 24 hours to arrest A549 cells at G2/M phase, the culture medium were refreshed and 7μM HPCPCB was added. The HPCPCB-treated cells were collected at various time points and analyzed for the cell cycle distribution. The results indicated that HPCPCB-induced G1 arrest (Figure 1A).

Furthermore, we performed Western blot to characterize the molecular mechanism of HPCPCB-induced G1 arrest (Figure 1B). The expression level of cell cycle promoting proteins such as CDC25A, CDK4, and Cyclin E were increased at 12 hr after A549 releasing from nocodazole arrest in DMSO solvent control group. In contrast, HPCPCB treatment significantly reduced or delayed the expression level of these cell cycle promoting proteins. Moreover, the cell cycle inhibiting protein p21 were up-regulated upon HPCPCB treatment.

**HPCPCB induces protein acetylation**

![Figure 1. HPCPCB induced cell cycle G1 phase arrest.](image)

(A) Flow cytometry demonstrating the induction of G1 phase accumulation by HPCPCB, whereas DMSO solvent control did not affect cell cycle. (B) Western blotting demonstrated that HPCPCB treatment abolished or delayed the change in expression level of cell cycle controlling proteins CDC25A, CDK4, and Cyclin E, whereas increased the level of p21 cell cycle inhibitor. *, P<0.05; **, P<0.01.

To determine whether HPCPCB could induce the acetylation of histone and non-histone proteins, Western blot analysis was performed. The data indicated that HPCPCB induced acetylation of histones H3 and H4 at a dose of 1/6 IC50 in 30 min (Figure 2A and 2B).

Furthermore, HPCPCB induced acetylation of non-histone proteins such as p53 and tubulin in 2 hours (Figure 3). HPCPCB treatment increased p21 expression in both A549 (p53 wild-type) and H1299 (p53 null) cells (Figure 3).

**HPCPCB inhibits HDAC activity**

The HDAC activity assay was performed to measure the HDAC inhibition ability of HPCPCB in vitro. As shown in Figure 4, HPCPCB efficiently inhibited HDAC activity at 2μM and 5μM.

### Table 1. Cytotoxicity of HPCPCB on normal human lung cells, normal breast cells, various lung carcinoma cells, and breast cancer cells.

<table>
<thead>
<tr>
<th></th>
<th>Lung (μM)</th>
<th>Breast (μM)</th>
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<tbody>
<tr>
<td>A549</td>
<td>6.94</td>
<td>MDAMB-231</td>
</tr>
<tr>
<td>H460</td>
<td>9.65</td>
<td>MCF-10A*</td>
</tr>
<tr>
<td>H1299</td>
<td>6.91</td>
<td>IMR90*</td>
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*, normal lung cells; *, normal breast cells.
Discussion

In this study, we developed a novel compound, which is derived from SAHA, named HPCPCB. We tested the cancer cell-specific cytotoxicity of HPCPCB and demonstrated that HPCPCB exhibited cytotoxicity toward cancer cells but not normal cells. Moreover, HPCPCB induced cell cycle arrest through reducing the expression of CDC25A, CDK4 and CyclinE proteins and upregulating the expression of the cell cycle inhibitor p21 protein. CDC25A promotes cell cycle progression by activating G1 CDK proteins such as CDK2 (Zou et al., 2001) and the degradation of CDC25A leads cells G1 arrest (Huang et al., 2011). p21 protein can bind to both the CDK4/cyclin D and CDK2/cyclin E complexes and inhibit both of their activity and lead cells to G1 phase arrest (He et al., 2005). Our studies suggest that HPCPCB broadly inhibited cancer cell growth via arresting cancer cells at G1 phase and deregulating the cell cycle control proteins.

The HDAC inhibition ability of HPCPCB was confirmed through the acetylation of histone and non-histone proteins in cell model and in 

in vitro assays in time- and dose-dependent manners. Non-histone proteins such as p53 and tubulin were acetylated after HPCPCB treatment. Previous studies show that the acetylation of p53 increases its protein stability and transcriptional activity (Bode and Dong, 2004; Minucci and Pelicci, 2006). The p53 activation is important in cell cycle G1 arrest. However, HPCPCB induced the expression of p21 protein in A549 and H1299 cells despite the p53 status, suggesting that activation of p21 involved changes in promoter-associated proteins, including HDACs, and not via p53-dependent transcriptional activation. Note that HPCPCB induced the acetylation of tubulin protein. Acetylation of tubulin is important for microtubule stabilization, and decreases cell motility (Palazzo et al., 2003; Xu et al., 2007). The tubulin acetylation implied that HPCPCB may inhibit cancer cell motility.

Compare to other HDAC inhibitors such as SAHA and OSU-HDAC-44 (Tang et al., 2010), HPCPCB similarly induced the acetylation of histone proteins H3 and H4 and non-histone protein p53 in A549 and H1299 cells. However, HPCPCB induced A549 G1 phase arrest instead of G2/M arrest. The difference may because HPCPCB...
preferentially deregulates the G1 cell cycle control proteins.

In conclusion, our studies show that HPCPCB broadly inhibited cancer cell growth and arrest cancer cells at G1 phase. The inhibition of HDAC activity in vitro and the induction of protein acetylation in vivo suggested that HPCPCB is a pan-HDAC inhibitor and induces acetylation of histone and non-histone proteins. We will further investigate whether prolonged treatment will induce apoptosis by HPCPCB. Moreover, the motility inhibition of cancer cells by HPCPCB will be examined. Animal models will be used to validate the antitumor and anti-metastasis effect of HPCPCB in vivo.

References


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新穎組蛋白去乙醯酶抑制劑的抑癌能力探討

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摘 要

前人研究發現，組蛋白去乙醯酶 (Histone deacetylases, HDACs) 在許多癌症腫瘤中有過量表現或過度活化的現象，因此 HDACs 為具有潛力的癌症治療標靶。在現行的血液腫瘤臨床治療上，組蛋白去乙醯酶抑制劑 (HDACi) 已證實具有多重抑癌能力；然而，HDACi 對於固體腫瘤的療效仍待進一步研究。本篇研究提出一個新穎的 HDACi ─ N-hydroxy-4-((1R,2R)-2-phenylcyclopropanecarboxamido) benzamide (HPCPCB) 以探討其作為治療固體腫瘤的可行性。我們首先利用多種肺癌、乳癌細胞株和正常肺細胞、乳房細胞測試 HPCPCB 的細胞毒性和專一性；接著利用流式細胞儀 (Flow cytometry) 觀測到 HPCPCB 處理過的細胞，會有細胞週期 G1 期停滯的現象。從西方點墨法 (Western blot) 發現 HPCPCB 會抑制或延遲 G1 細胞週期調控蛋白 CDC25A、CDK4 及 cyclin E 的表現量，並且提高細胞週期抑制蛋白 p21 的表現量。此外，我們更進一步證明，HPCPCB 會抑制 HDAC 的活性，並會引起組蛋白 H3 及 H4，和非組蛋白 p53 及 tubulin 的乙醯化程度增加。我們的實驗結果顯示，HPCPCB 是一有效的 HDAC 抑制劑，並有進一步作為癌症治療用藥的研究可行性。

關鍵詞：組蛋白去乙醯酶、乙醯化、癌症、細胞週期停滯