A Novel Histone Deacetylase Inhibitor Exhibits Antitumor Activity via Apoptosis Induction, F-Actin Disruption and Gene Acetylation in Lung Cancer

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Abstract

**Background:** Lung cancer is the leading cause of cancer mortality worldwide, yet the therapeutic strategy for advanced non-small cell lung cancer (NSCLC) is limitedly effective. In addition, validated histone deacetylase (HDAC) inhibitors for the treatment of solid tumors remain to be developed. Here, we propose a novel HDAC inhibitor, OSU-HDAC-44, as a chemotherapeutic drug for NSCLC.

**Methodology/Principal Findings:** The cytotoxic effect of OSU-HDAC-44 was examined in three human NSCLC cell lines including A549 (p53 wild-type), H1299 (p53 null), and CL1-1 (p53 mutant). The antiproliferative mechanisms of OSU-HDAC-44 were investigated by flow cytometric cell cycle analysis, apoptosis assays and genome-wide chromatin-immunoprecipitation-on-chip (ChIP-on-chip) analysis. Mice with established A549 tumor xenograft were treated with OSU-HDAC-44 or vehicle control and were used to evaluate effects on tumor growth, cytokinesis inhibition and apoptosis. OSU-HDAC-44 was a pan-HDAC inhibitor and exhibits 3–4 times more effectiveness than suberoylanilide hydroxamic acid (SAHA) in suppressing cell viability in various NSCLC cell lines. Upon OSU-HDAC-44 treatment, cytokinesis was inhibited and subsequently led to mitochondria-mediated apoptosis. The cytokinesis inhibition resulted from OSU-HDAC-44-mediated degradation of mitosis and cytokinesis regulators Auroraa B and survivin. The deregulation of F-actin dynamics induced by OSU-HDAC-44 was associated with reduction in RhoA activity resulting from srGAP1 induction. ChIP-on-chip analysis revealed that OSU-HDAC-44 induced chromatin loosening and facilitated transcription of genes involved in crucial signaling pathways such as apoptosis, axon guidance and protein ubiquitination. Finally, OSU-HDAC-44 efficiently inhibited A549 xenograft tumor growth and induced acetylation of histone and non-histone proteins and apoptosis in vivo.

**Conclusions/Significance:** OSU-HDAC-44 significantly suppresses tumor growth via induction of cytokinesis defect and intrinsic apoptosis in preclinical models of NSCLC. Our data provide compelling evidence that OSU-HDAC-44 is a potent HDAC targeted inhibitor and can be tested for NSCLC chemotherapy.

Introduction

Lung cancer is the leading cause of cancer mortality worldwide. The 5-year overall survival of non-small cell lung cancer (NSCLC) is less than 15% in many countries [1,2]. The standard therapeutic strategy for advanced NSCLC is platinum-based double-agent chemotherapy which, however, has reached a plateau of potency in improving survival of patients [3,4]. Only a few “target agents” have showed benefits when used in combination with platinum-based double-agent for NSCLC chemotherapy, such as bevacizumab, erlotinib and gefitinib, in a subset of patients [5,6]. Therefore, the development of novel molecular targeted drugs with more general effectiveness for lung cancer patients is an imperative task.

The epigenetic changes as well as genetic alterations are associated with tumorigenesis [7]. A recent report identifies that the epigenetic changes involving modifications of histones H2A and H3 in NSCLC patients influence the overall survival and disease-free survival, providing the prognostic value of histone modifications [8]. It also reveals the rationale for the use of drugs against histone modification as a therapeutic strategy for NSCLC.

Histone deacetylases (HDACs) are the enzymes that catalyze the deacetylation of histones and epigenetically regulate chromatin architecture and gene expression. It has been demonstrated that...
inhibition of HDACs reverses aberrant epigenetic status and exhibits potent antitumor activities by inducing cell cycle arrest, differentiation and/or apoptosis in diverse cancer cells [9,10]. HDAC inhibitors are classified into six groups according to their chemical structures and at least 12 of them have progressed to clinical trials [9,11]. To date, the U.S. Food and Drug Administration approves two HDAC inhibitors, vorinostat (SAHA, suberoylanilide hydroxamic acid, Zolinza®) and romidepsin (FK228, depsipeptide, Istodax®), for the treatment of cutaneous manifestations of cutaneous T-cell lymphoma (CTCL) [12]. However, some adverse events occur in patients treated with vorinostat or other HDAC inhibitors, which may result from the high concentrations of dose used during the treatment for solid tumors in clinical trials [11,13].

In the present study, we propose a novel class of potent phenylbutyrate-based HDAC inhibitor, OSU-HDAC-44 [4-(2,2-dimethyl-4-phenyl-butyrylamino)-N-hydroxy-benzamide], a derivative of known HDAC inhibitor, N-Hydroxy-4-(4-phenylbutyryl-amino)benzamide (HTPB) [14]. The antitumor activities and mechanisms of OSU-HDAC-44 were studied in NSCLC cell and mice xenograft models. We found that OSU-HDAC-44 was a pan-HDAC inhibitor and exhibited 3-4 times more effectiveness in suppressing cell proliferation in vitro and tumor growth in vivo compared to SAHA or trichostatin A (TSA). In addition, OSU-HDAC-44 induced mitosis and cytokinesis defect followed by mitochondria-mediated apoptosis in both cell and animal models. Chromatin-immunoprecipitation-on-chip analysis revealed the genome-wide target genes which were induced by OSU-HDAC-44-mediated hyperacetylation of chromatin. Our data suggest that OSU-HDAC-44 was an HDAC inhibitor and could be applied as targeted anticancer drug for NSCLC chemotherapy.

Results
OSU-HDAC-44 inhibits cell proliferation and shows synergistic effects with cisplatin regardless of p53 status

The structure of OSU-HDAC-44 and SAHA are shown in Fig. 1A. Docking analysis demonstrated that OSU-HDAC-44 interacted with the catalytic domain of HDAC 8, suggesting the direct function of OSU-HDAC-44 in targeting HDACs (Fig. 1B).

The cell growth inhibition activities of OSU-HDAC-44 were assessed in three human NSCLC cell lines including A549 (p53 wild-type), H1299 (p53 null), and CL1-1 (p53 mutant). SAHA was included as a positive control HDAC inhibitor. OSU-HDAC-44 significantly inhibited cell proliferation in all cancer cell lines despite their differences in p53 background, and did not cause

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**Figure 1. Chemical structure, molecular docking analysis, and the effect of OSU-HDAC-44 on cell viability**

(A) Chemical structure of OSU-HDAC-44 and SAHA. (B) Molecular docking analysis of OSU-HDAC-44 and SAHA. The structures of OSU-HDAC-44 and SAHA were calculated and the docking mode on catalytic domain of HDAC8 was predicted using the docking program GOLD 4.0.1. (C) Dose-dependent effects of OSU-HDAC-44 (left) and SAHA (right) on cell viability in IMR90, H1299, A549 and CL1-1 cells. Cells were treated with 0.5–10 μM of OSU-HDAC-44 or SAHA for 48 h, and cell viability was assessed by trypan blue exclusion assay. (D) OSU-HDAC-44 synergized with cisplatin to suppress cell proliferation. Cells were exposed to cisplatin (Cis) alone for 4 h, OSU-HDAC-44 (HDAC-44) alone for 48 h, or pretreated with OSU-HDAC-44 for 48 h before cisplatin treatment for 4 h, and then drug were withdrew and cells were incubated with drug-free media for additional 48 h. Cell viability was assessed by trypan blue exclusion assay. CL1-1 cells were treated with 4.4 μM cisplatin or 0.3 μM OSU-HDAC-44, A549 cells were treated with 1.6 μM cisplatin or 0.2 μM OSU-HDAC-44. Data represent mean ± SEM from three independent experiments. * P<0.05; ** P<0.01; *** P<0.001.

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OSU-HDAC-44 inhibits lung cancer

OSU-HDAC-44 induces cytokinesis inhibition and apoptosis

To investigate the underlying mechanism of cell growth repression by OSU-HDAC-44, the effects of OSU-HDAC-44 on cell cycle progression were assessed by flow cytometry. Treatment with 2.5 μM OSU-HDAC-44 for 24 hours caused A549 and H1299 cells to accumulate in G2/M phase (4N cells), and subsequently led to apoptosis (sub-G1 cells) at 48 hours treatment, while exposure to higher concentration (5 μM) of SAHA for 48 hours had similar effect (Fig. 2A), indicating that OSU-HDAC-44 exerted a more potent cell cycle deregulation effect than did SAHA. To examine the cellular consequences of OSU-HDAC-44-mediated accumulation of 4N cells, time-lapse microscopic analyses were performed. As shown in Fig. S1A, OSU-HDAC-44 caused the appearance of the defective cleavage furrow structure and the two daughter cells were fused back together, while untreated cells passed normally through cell division. Concordantly, about 20% cells treated with OSU-HDAC-44 were accumulated as bi-nucleated cells, compared with less than 5% of control cells (Fig. 2B and Fig. S1B); OSU-HDAC-44 also caused micronuclei formation and disrupted the normal structure of F-actin of A549 and H1299 cells (Fig. 2B). Hence, these results suggested that OSU-HDAC-44 may cause aberrant cytokinesis and subsequently led to apoptosis in lung cancer cells.

To identify the molecular mechanism involved in OSU-HDAC-44 induced cytokinesis inhibition, the cell cycle-regulatory proteins were examined. The oscillation of mitotic inhibitor Weel and mitotic markers phosphorylated histone H3 and cyclin B expression indicated that OSU-HDAC-44-treated cells were in M phase after 12 hours treatment and subsequently exited M phase (Fig. S1C), accompanied with cytokinesis defect. Moreover, OSU-HDAC-44 caused decreases in protein levels of Aurora B and survivin (Fig. 2C; upper), which are essential for the progression of mitosis and cytokinesis [15,16]. Notably, OSU-HDAC-44 induced ubiquitination of Aurora B and survivin, and cotreatment with proteasome inhibitor MG132 prevented the OSU-HDAC-44-induced degradation of Aurora B and survivin (Fig. 2C; middle and lower). Next, we used nocodazole to synchronize cells at pre-metaphase and to further confirm that OSU-HDAC-44 indeed triggered abnormal degradation of Aurora B and survivin at mitotic phase. As shown in Fig. S1D and E, treatment with nocodazole for 24 hours caused accumulation in Aurora B and survivin proteins, whereas combination of OSU-HDAC-44 and nocodazole resulted in decreases Aurora B and survivin protein levels upon 24 hours post-treatment. These results suggested that the OSU-HDAC-44-mediated failure of cytokinesis may partly result from the downregulation of Aurora B and survivin proteins via 26S proteasome pathway.

OSU-HDAC-44 activates the intrinsic apoptotic pathway

To further elucidate the OSU-HDAC-44-induced apoptosis, we performed phosphatidylinositol (PS) staining analyses to detect the early process of apoptosis. As shown in Fig. S2, OSU-HDAC-44 treatment for 24 hours increased the intensity of PS staining in contrast to low staining intensity upon DMSO treatment in A549 and H1299 cells. In addition, OSU-HDAC-44 treatment significantly stimulated caspase-3 and caspase-9 (an indicator of the intrinsic mitochondrial pathway) activities after 24 hours treatment whereas the activity of caspase-8 (an indicator of the extrinsic membrane receptor pathway) remained unaffected in A549 and H1299 cells (Fig. 2D, left). Moreover, treatment with 2.5 μM OSU-HDAC-44 for 12 hours caused a decrease in anti-apoptotic protein Bcl-xl, while it increased the pro-apoptotic protein Bax, within 6-12 hours treatment in A549 and H1299 cells (Fig. 2D, right). The release of cytochrome c into the cytosol accompanied by the cleavage of pro-caspase-9 was also seen after OSU-HDAC-44 treatment for 24-48 hours. These results further confirmed that OSU-HDAC-44 could induce the intrinsic apoptotic pathway in lung cancer cells.

OSU-HDAC-44 induces protein acetylation with its ability to target numerous HDACs

The biomarkers of HDAC inhibition are acetylation of histone and non-histone proteins, and induction p21<sup>CIP</sup> expression in a p53-independent manner [17,18]. Exposure to OSU-HDAC-44 induced acetylation of histone H3, histone H4 and p53 in a dose-dependent manner (Fig. 3A) and time-dependent manner (Fig. 3B), while it did not affect the HDAC1 and HDAC6 protein levels (Fig. 3B and Fig. S3). Notably, such effects were greater compared to that of SAHA. Despite the p53 status, OSU-HDAC-44 induced the expression of p21<sup>CIP</sup> mRNA and protein in A549 and H1299 cells (Fig. S4). To examine the target specificity of OSU-HDAC-44 on class I, II, and IV HDACs, in vitro HDAC inhibition assay was performed. As shown in Fig. 3C, the deacetylase activities of different HDAC isotypes including class I (HDAC1 and HDAC2), class II (HDAC4 and HDAC6), and class IV (HDAC11), were significantly inhibited by OSU-HDAC-44. Such effects were greater compared to that of SAHA, a known pan-HDAC inhibitor. These results suggested that OSU-HDAC-44 induced protein acetylation by exerting broad inhibitory activity upon numerous HDACs.

OSU-HDAC-44 increases gene expression by loosening the chromatin structure

To determine the direct effects of OSU-HDAC-44 on chromatin structure and gene expression, the chromatin-immunoprecipitation (ChIP) on-chip analysis was performed using the antibody against the loose chromatin mark, acetylated lysines 9 and 14 of histone H3 (H3K9K14Ac), after 2.5 μM OSU-HDAC-44 treatment for 2 hours in A549 and H1299 cells. Induction of histone acetylation in 33 common gene loci of A549 and H1299 were identified after OSU-HDAC-44 treatment (Table S1). Several of these 33 genes had been demonstrated to play important roles in certain signaling pathways, such as apoptosis, oxidative stress response, axon guidance and protein ubiquitination pathways (Table 1). To confirm microarray data, we validated the chromatin structure of some of the gene loci including srGAP1, NRA11 and FOXO4 by ChIP-PCR using the antibody against H3K9K14Ac. As shown in Fig. 4A, treatment with 2.5 μM OSU-HDAC-44 for 2 hours increased the amount of srGAP1, NRA11 and FOXO4 promoter DNA with loose chromatin structure compared to untreated cells. Concordantly, the mRNA levels of srGAP1, NRA11 and FOXO4 were increased after OSU-HDAC-44 treatment for 24 hours (Fig. 4B).

OSU-HDAC-44 down-regulates F-actin dynamics via srGAP1 induction

OSU-HDAC-44 treatment induced F-actin aggregation (Fig. 2B). Previous study has indicated that srGAP1 binds to
Figure 2. OSU-HDAC-44 induces cytokinesis inhibition and subsequently leads to intrinsic apoptosis. (A) The effects of OSU-HDAC-44 on cell cycle distribution in A549 and H1299 cells. Cells were treated with 2.5 μM OSU-HDAC-44 or 5 μM SAHA for indicated times and assessed by flow cytometry. Left, results from one representative experiment are shown. Right, the mean percentage of G2/M and sub-G1 fraction population is...
the active forms of RhoA and Cdc42 and inhibits their activities in regulating actin polymerization in neuron cells [19]. However, the biological function of srGAP1 binding to RhoA remains unclear in other cells. Using immunoprecipitation (IP)-Western, we showed that OSU-HDAC-44 increased the interaction between srGAP1 and RhoA in A549 lung cancer cells (Fig. 4C). Interestingly, knockdown of srGAP1 not only abolished the OSU-HDAC-44-mediated decrease in RhoA-GTP level (Fig. 4D, upper), but also restored the dynamics of F-actin after OSU-HDAC-44 treatment (Fig. 4D, lower). These results indicated that OSU-HDAC-44 down-regulated RhoA activity partly via srGAP1 induction, leading to destruction of normal F-actin fibers.

OSU-HDAC-44 inhibits lung tumor xenograft growth in vivo

To further evaluate the antitumor activity of OSU-HDAC-44, Bulb/c null mice bearing A549 lung tumor xenograft were injected intraperitoneally with 7.5–30 mg/kg of OSU-HDAC-44, 3 days/week for three weeks. TSA of 0.5 mg/kg, which has been demonstrated to exhibit anti-tumor growth effects in xenograft of breast and bladder cancer cells [20,21], was used as a positive control drug. As shown in Fig. 5A, treatment with 7.5, 15 and 30 mg/kg OSU-HDAC-44 significantly inhibited tumor growth by 62%, 78% and 90%, respectively, on day 33 post-treatment compared with vehicle control. Treatment with OSU-HDAC-44 did not adversely affect body weight and caused no detectable

Figure 3. Effect of OSU-HDAC-44 on the biomarkers associated with broad inhibition on numerous HDACs. Dose-dependent effects (A) and time-dependent effects (B) of OSU-HDAC-44 on the histone and non-histone proteins. Ac-H3, acetylated histone H3; Ac-H4, acetylated histone H4; Ac-p53, acetylated p53; p53, total p53. (C) OSU-HDAC-44 suppressed activities of class I (HDAC1 and HDAC8), class II (HDAC4 and HDAC6), and class IV (HDAC11) HDACs. Different HDAC isotypes were immunoprecipitated from H1299 nuclear extract by specific antibodies, and then subjected to in vitro HDAC inhibition assay as described in Materials and Methods section. Data represent mean ± SEM from three independent experiments. ** P<0.01; *** P<0.001. doi:10.1371/journal.pone.0012417.g003
OSU-HDAC-44 induces protein acetylation, apoptosis and cytokinesis inhibition

To confirm that OSU-HDAC-44 suppressed xenograft tumor growth via targeting the HDACs and inducing apoptosis in vivo, mice bearing established A549 tumors were treated with a single dose of OSU-HDAC-44. After treatment for indicated time, tumors were dissected and subjected to Western blot, immunohistochemistry or fluorescence immunohistochemistry analysis (Fig. 5B–D). Acetylation of histone H3, histone H4 and p53 were profoundly increased after 2 hours treatment. The protein levels of Bcl-xL and survivin started to decrease after 2 hours treatment, while the level of Bad protein was increased after 4 hours treatment (Fig. 5B). Activated caspase-3 was also detected in both the cytosol and nucleus after 8 hours treatment and was further enhanced after 24 hours treatment (Fig. 5C). Furthermore, OSU-HDAC-44 decreased Aurora B levels and interrupted its association with metaphase chromosome in comparison with DMSO control cells (Fig. 5D). These results demonstrated that OSU-HDAC-44 could induce apoptosis and down-regulate mitotic and cytokinesis regulators, Aurora B and survivin, in vivo. In addition, increase of HDAC inhibition biomarkers such as acetylation of histone H3, histone H4 and p53 was evident in tumors of treated mice.

Discussion

Since HDACs are promising targets for cancer therapy, a number of HDAC inhibitors are in clinical trials as single therapy and/or in combination with other anticancer drugs [9]. However, effective HDAC inhibitors for treatment of solid tumors remain to be developed. In this study, we provide compelling evidence from cell and animal studies that OSU-HDAC-44, a phenylbutyrate-based compound, is a potential HDAC inhibitor for NSCLC treatment. OSU-HDAC-44 targeted numerous members within three classes of HDACs in vitro and efficiently stimulated protein acetylation in cell and animal models (Fig. 3 and 5). OSU-HDAC-44 repressed cell viability and induced apoptosis in various NSCLC cell lines with 3–4 times greater potency than SAHA (Fig. 1C and 2A). In addition, submicromolar concentration of OSU-HDAC-44 exhibited prominently synergistic effects in combination with cisplatin on suppressing proliferation of NSCLC cell lines (Fig. 1D). The xenograft experiments further confirmed that OSU-HDAC-44 induced cell apoptosis and thereby inhibited tumor growth in vivo (Fig. 5) without adversely affected body weight, major organs and hematological parameters (Fig. 6). Collectively, these results suggested that OSU-HDAC-44 is a promising candidate HDAC inhibitor for NSCLC treatment.

It has been shown that several kinases and regulatory proteins, such as Aurora B, survivin as well as small GTPase RhoA are required to complete cytokinesis [22]. Inhibition of Aurora B or depletion of survivin can prevent the late steps of cytokinesis, leading to formation of multi-nucleated cells [15,16]. In the current study, we provided evidence that OSU-HDAC-44 induced proteolysis of Aurora B and survivin both in vitro and in vivo (Fig. 2C and Fig. 5B, D), which was associated with OSU-HDAC-44-mediated cytokinesis inhibition, resulting in the accumulation of bi-nucleated cells (Fig. 2B and Fig. S1A–B). In addition, combination of a pre-metaphase inducer nocodazole and OSU-HDAC-44 resulted in decrease of Aurora B and survivin protein levels upon 24 h post-treatment (Fig. S1E). These data suggested that OSU-HDAC-44-mediated cytokinesis defect was due to abnormal degradation of Aurora B and survivin in mitotic phase. It has been reported that overexpression of Aurora B correlates with survivin expression in the nucleus, lymph node invasion, and poor prognosis in NSCLC patients [23]. Thus, the clinical efficacy of OSU-HDAC-44 in relation to down-regulated Aurora B and survivin in treatment of NSCLC patients is worthy of further investigation.
Interestingly, we found that OSU-HDAC-44 decreased the activity of a small GTPase RhoA via induction of srGAP1 and contributed to dysregulation of F-actin dynamics (Fig. 4C, D).

Similar to our finding of selective chromatin change of a fraction of gene loci in ChIP-on-chip, recent studies using cDNA microarrays indicate that several HDAC inhibitors such as TSA, SAHA, MS-275 and depsipeptide alter only 7–20% gene expressions in various cancer cell lines [28–30]. Specific recruitment of corepressor complexes containing HDACs by transcription factors and/or transcription regulators is believed to play an essential role in transcriptional repression [31–33], however, the selective action of HDAC inhibitors on specific genes remains unclear. Thus, it is worthy to investigate whether there may be common and critical transcription-regulatory complexes containing HDACs that determine the acetylation levels of chromatin of these genes validated from ChIP-on-chip data.

In conclusion, our findings show that OSU-HDAC-44 is a novel pan-HDAC inhibitor that exhibits a broad spectrum of antitumor activities in NSCLC cell and xenograft models, which involves not only histone acetylation-dependent activation of gene transcription, but also activation of intrinsic apoptotic pathways and post-translational down-regulation of mitotic regulators.

Figure 4. OSU-HDAC-44 decreased RhoA activity via srGAP1 induction, leading to F-actin dysregulation. (A) Chromatin-immunoprecipitation-PCR analyses confirmed that treatment with 2.5 μM OSU-HDAC-44 for 2 h induced acetylation of histone H3 (H3K9K14Ac) in the promoter region of srGAP1, NR4A1 and FOXO4 genes. (B) OSU-HDAC-44 increased the mRNA levels of srGAP1, NR4A1 and FOXO4 genes using real-time RT-PCR analyses. Cells were treated with 2.5 μM OSU-HDAC-44 for 24 h and total RNA was extracted for the real-time RT-PCR analyses. Data represent mean ± SEM from three independent experiments. *P<0.05. (C) Immunoprecipitation assay indicated that increased interaction between srGAP1 and RhoA was induced by OSU-HDAC-44. A549 cells were treated or untreated with 2.5 μM OSU-HDAC-44 for 24 h and subjected to IP-Western analyses. (D) si-srGAP1 abrogated the OSU-HDAC-44-induced decrease in RhoA activity (upper) and rescued the normal structure of F-actin after OSU-HDAC-44 treatment (lower). A549 cells transfected with srGAP1 siRNA were treated with 2.5 μM OSU-HDAC-44 for 24 h and subjected to RhoA activation assay and immunofluorescence analyses. Scale bars: 30 μm.

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Aurora B and survivin. In addition, RhoA/F-actin motility control was inhibited by srGAP1 and several apoptosis induction proteins were activated by OSU-HDAC-44 (Fig. 7). Collectively, our data provide compelling evidence that OSU-HDAC-44 is an HDAC targeted inhibitor and has the potential to be tested for NSCLC treatment and combination chemotherapy.

Materials and Methods

Cell lines and culture conditions

Human normal lung cell line IMR90 and human NSCLC cell lines A549 and H1299 were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and the human NSCLC cell line CL1-1 was kindly provided by Dr. P-C Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan) [34]. All cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS) (BIOCHROM AG, Leonorenstr, Berlin, Germany) and 1% penicillin-streptomycin (GIBCO) and incubated at 37°C in 5% CO2 atmosphere.

Preparation of OSU-HDAC-44

Isobutyric acid (1.4 mL) was added dropwise to a mixture of diisopropylamine (2.2 mL, 0.015 mol) and 54% sodium hydride in mineral oil (0.74 g, 0.0165 mol) in THF (40 mL), and refluxed for 15 min. When the solution was cooled to 0°C, a standard solution of n-butyllithium in heptane (1.45 mmol/mL; 9.4 mL) was added. After 20 min at 0°C, the mixture was heated to 30–35°C for 30 min and then cooled to 0°C when (2-bromoethyl)-benzene (2.8 mL, 15 mmol) was added to the reaction mixture over 20 min. The ice-bath was retained for 30 min, the mixture was then heated to 30–35°C for 1 h, and then 40 mL of water was added to the reaction mixture at a temperature below 15°C. The aqueous layer was separated, and the organic layer was washed...
with a mixture of 20 mL of water and 30 mL of ethyl ether. Aqueous layers were combined, back extracted with 20 mL of ethyl ether then acidified with 1N hydrochloric acid and the product was extracted with 30 mL of ethyl ether twice. The combined organic layer was washed with 20 mL of saturated brine, dried with Na2SO4, and concentrated under vacuum. Hexane was added to the resulting colorless oil to yield 1.1 g of white solid 2, 2,-Dimethyl-4-phenylbutyric acid compound. Oxalyl chloride (2 mmol) was added to the cooled solution of 2, 2,-Dimethyl-4-phenylbutyric acid (1 mmol) in dichloromethane (5 mL), and the reaction mixture was then brought to room temperature and stirred for 4 h. After the completion of the reaction, solvent was removed under vacuum. The residue was dissolved in dichloromethane (10 mL) and cooled to 0°C. Paraamino benzoic acid was then added to the reaction mixture followed by addition of triethyl amine. Resultant mixture was brought to room temperature and stirred overnight. Reaction mixture was then concentrated and purified by column chromatography to give 4-(2, 2-dimethyl-4-phenylbutanamido) benzoic acid compound. The cooled 4-(2, 2-dimethyl-4-phenylbutanamido) benzoic acid compound (1 mmol) in DMF (1 mL) was added triethyl amine (1.2 mmol) followed by PyBOP (1.2 mmol). Resultant mixture was stirred at room temperature for 4 h. After complete consumption of starting material as evidenced by TLC, reaction mixture was cooled to 0°C and hydroxylamine hydrochloride (1.2 mmol) was added to the reaction mixture followed by addition of triethyl amine (1.5 mmol). Resultant mixture was stirred at room temperature overnight and then quenched with water. Solid was filtered and purified by column chromatography to give OSU-HDAC-44.

Analysis of cell viability

Cells were seeded in 6-well plates and treated with various concentrations of OSU-HDAC-44 or SAHA for 48 h, then stained with Trypan Blue solution (0.4%) (Sigma-Aldrich, St. Louis, MO) to measure their effects on cell proliferation. For its synergistic effect in combination with cisplatin (Bristol-Myers Squibb Caribbean Company, New York, NY), CL1-1 and A549 cells were exposed to cisplatin alone for 4 h, OSU-HDAC-44 alone for 48 h, or pretreated with OSU-HDAC-44 for 48 h before cisplatin treatment for 4 h, and then drug-containing media were replaced by drug-free media. Treated cells were incubated for additional 48 h and cell viability was assessed by Trypan Blue exclusion assay. The concentrations of drugs were described as follows: CL1-1 cells were treated with 4.4 μM cisplatin and/or 0.3 μM OSU-HDAC-44; A549 cells were treated with 1.6 μM cisplatin and/or 0.2 μM OSU-HDAC-44. For elucidation of the OSU-HDAC-44-induced cell death, phosphatidylserine (PS) staining analyses were performed and described in the Supplementary Methods S1.

Cell cycle analysis

Cell cycle distribution was determined by flow cytometry. Cells (2×10^6) were treated with 2.5 μM OSU-HDAC-44 or 5 μM SAHA for 24 or 48 h. Cells were trypsinized and fixed with 70% ethanol for at least 2 h at −20°C. Fixed cells were stained with a solution containing 20 μg/ml propidium iodide, 200 μg/ml RNase A, and 0.1% Triton X-100 for 20 minutes in the dark. Cell cycle distribution was performed by FACSscan flow cytometry (BD Biosciences, Mountain View, CA) and calculated using ModFIT LT 2.0 version software (BD Biosciences). For examina-
tion of the cellular consequences of OSU-HDAC-44-mediated accumulation of 4N cells, time-lapse microscopic analyses were performed and described in the Supplementary Methods S1.

Caspase activity assay

Caspase activity was measured with the caspase luminescent assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, cells were plated in a 96-well plates and treated with 2.5 μM OSU-HDAC-44 for 12 or 24 h, followed by incubating with various synthetic caspase substrates (Ac-DEVD-pNA, Ac-LETD-pNA, and Ac-LEHD-pNA) to measure the activity of caspases 2, 3, 8, and 9, respectively. After incubation for 1 h, luminescence was detected on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

siRNA transfection

The srGAP1 siRNAs were purchased from Invitrogen (Carlsbad, CA). Cells were transfected with 300 nM of srGAP1 siRNA duplexes (sense, 5’- AAA CGU AUC AUC CAU AUC CUG CAC C -3’ and antisense: 5’- GGU GCA GGA UAU GGA UGA UAC GUU U -3’) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. After transfection for 48 h, the cells were subjected to OSU-HDAC-44 treatment.

Western blot analysis

Cells were lysed on ice using RIPA buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonfluoride, 5 μg/ml leupeptin, 10 μg/ml aprotonin). The lysate was centrifuged at 13000 r.p.m for 15 minutes at 4°C. Protein extracts were solubilized in SDSPAGE loading buffer (60 mM Tris-base, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol). Samples containing equal amounts of protein (50 μg) were separated on an 8% SDS-PAGE and electroblotted onto Immobilon-P membranes (Millipore Co., Bedford, MA) in transfer buffer. Immunoblotting was performed for various proteins, using the conditions described in the Table S2, available online. Antibody reaction was visualized using Western blot chemiluminescence reagent (Millipore).

Figure 7. The antitumor activity of OSU-HDAC-44 via cytokinesis defect, F-actin disruption, apoptosis induction, and gene acetylation. OSU-HDAC-44 is a novel pan-HDAC inhibitor that exhibits a broad spectrum of antitumor activities in NSCLC cell and xenograft models, which involves histone acetylation-dependent activation of gene transcription in nucleus. For example, re-expression of NR4A1 and FOXO4 along with caspase activation induces intrinsic apoptosis. In addition, RhoA/F-actin motility control is inhibited by srGAP1 resulting from activation by OSU-HDAC-44. OSU-HDAC-44 also induces post-translational down-regulation of mitotic regulators, Aurora B and survivin leading to cytokinesis defect and apoptosis. doi:10.1371/journal.pone.0012417.g007

Immunoprecipitation assay

Catch and Release Reversible Immunoprecipitation System kit (Upstate, Temecula, CA) was used for protein-protein interaction analysis. One mg cell protein lysates were incubated with the appropriate antibodies, including anti-srGAP1, anti-RhoA, anti-Aurora B, anti-survivin or normal mouse-IgG, and 10 μl affinity ligand, and immunoprecipitation was then performed according to the manufacturer’s protocol. After incubation at 4°C overnight, immune complexes were washed with wash buffer for three times. Proteins were eluted and then blotted with appropriate antibodies using the conditions described in the Table S2.

RhoA activation assay

The RhoA activation assay was performed by using active Rho pull-down and detection Kit (Pierce, Rockford, IL). Briefly, a glutathione S-transferase (GST) fusion protein containing the Rho binding domain (RBD) from Rhotekin was used. One mg protein lysates were incubated with 400 μg of purified GST-Rhotekin-RBD immobilized on agarose-glutathione beads for 1 hour at 4°C with constant agitation. The beads were washed three times with 1X Lysis/Wash buffer and bound proteins were eluted and subjected to Western blot analysis using RhoA antibody described in the Table S2.

Molecular docking analysis

In order to show the interaction between OSU-HDAC-44 and HDAC, molecular docking assay was conducted. The reference compound, SAHA, was included. We calculated the structure of OSU-HDAC-44 and SAHA and predicted the docking mode on catalytic domain of HDAC8 using the docking program GOLD 4.0.1 to confirm the accuracy of this prediction program. The three dimensional structure of OSU-HDAC-44, the binding affinity of OSU-HDAC-44 to HDAC8, and the angles of OSU-HDAC-44 and HDAC8 were calculated by this prediction program, with consideration of molecular interaction, such as hydrogen bond and van der Waals force.
HDAC inhibition Assay

Different HDAC isoforms were immunoprecipitated from nuclear extract using specific anti-HDAC-1, −4, −6, −8, and −11 antibodies. The HDAC activity assay was performed using a HDAC fluorescent activity assay kit (BIOMOL Inc, Plymouth Meeting, PA) according to the manufacturer’s instructions. Briefly, the specific HDAC isoforms were added to the diluted OSU-HDAC-44 (1 μM) and SAHA (1 μM), and then the substrate was added. Samples were incubated for 10 min at 25 °C, followed by adding developer to stop the reaction. After incubation for 10 min, luminescence was recorded on a SpectraMax® M5 microplate reader (Molecular Devices, Sunnyvale, CA).

Target promoter chromatin immunoprecipitation (ChIP)-PCR Assay

Treated and untreated lung cancer cells were cross-linked with 1% formaldehyde for 15 min at 37 °C. Chromatin was immunoprecipitated with anti-acetylated lysine 9 and lysine 14 of histone H3 (H3K9K14Ac) using the ChIP assay kit (Upstate) according to the manufacturer’s instructions and the conditions were described in the Table S2. PCR analysis for protein-DNA complex was performed using the following primer pairs: sGAPI promoter, forward, 5′- TTT CCA TAC CAT CGC TTT CC-3′, and reverse, 5′- AAA CCC CTT CCT GAC GTG AG -3′; NR4A1 promoter, forward, 5′- GAC CTT CAG CAA GTG CCA TT -3′, and reverse, 5′- GCC CTT GAG ACG TGA AG -3′; FOXO4 promoter, forward, 5′- GGA GAG ATG GGT TCG ACC AT -3′, and reverse, 5′- TCT CCA ACG GCT TTA CTT CT -3′.

Chromatin structure profiling assay: ChIP-on-chip assay

The A549 and H1299 cells (4×10^6) were treated with DMSO or OSU-HDAC-44 for 2 h, and then immunoprecipitated using antibody to H3K9K14Ac as the conditions described in the Table S2. DNA was amplified and labeled by ligation-mediated PCR with Cy5 and Cy3 fluorescent dyes, respectively. Both pools of labeled DNA were hybridized to the NimbleGen human 385k array (Roche NimbleGen Inc., Madison, WI). Images of fluorescence intensities were generated by scanning using GenePix 4000B scanner (Axon Instruments, Union City, CA), and then data were extracted and ChIP signals were normalized using NimbleGen SignalMap software. The ChIP-on-chip data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE20304 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20304).

Real-time RT-PCR assay

Expression levels of sGAPI, NR4A1 and FOXO4 mRNA were assayed by real-time RT-PCR analysis using the GAPDH gene as an internal control. The primers used in real-time RT-PCR are as follows: sGAPI, forward, 5′- GGA TGG CCC TGT TTA TGA GA -3′ and reverse: 5′- CCG CCC AAC ATA GTA GAA AAA CT -3′; NR4A1, forward, 5′- GGC ATG GTG AAG GAA GTT GT -3′ and reverse: 5′- GCC TGG TGT AGA CCA GCT GA G -3′; FOXO4, forward, 5′- CTT TGC TGA GCC AGA TCC GTG AG -3′ and reverse: 5′- TTC CAA CAG CAT TGC TCA TC -3′; GAPDH, forward: 5′- AAT CCC ATC ACC ACC TTC CA -3′ and reverse: 5′- CCT GCT TCA CCA CCT TCT TG -3′. Relative quantitation using the comparative Ct method with the data from ABI PRISM 7000 (version 1.1 software) was performed according to the manufacturer’s protocol. Analysis of p27 gene expression and its primer sequence are described in the Supplementary Methods S1.

Immunofluorescence and confocal microscopic analysis

To stain for DNA and F-actin, the fixed cells were stained with DAPI and Phalloidin, respectively, for 1 h and then the images were recorded by an OLYMPUS FV1000 confocal microscope (Olympus America Inc., Melville, NY). For examination of the degradation of Aurora B and survivin at mitotic phase by OSU-HDAC-44, nocodazole was used to synchronize cell and then cells were subjected to immunofluorescence and confocal microscopic analysis as described in the Supplementary Methods S1.

Xenograft studies

Athymic nu/nu female mice (BALB/c, 4–5 weeks of age, were obtained from the National Laboratory Animal Center (Republic of China, Taiwan) after being approved by Institutional Animal Care and Use Committee (IACUC), National Cheng Kung University (IACUC Approval No. 99131) and maintained in pathogen free conditions. Eight mice per group were used in the xenograft studies. The animals were implanted subcutaneously with 5×10^6 A549 cells in 0.1 ml Hanks’ balanced salt solution (HBSS) in one flank per mouse. The tumor size was measured according to the formula: (Length×Width^2)/2. When tumors had attained a mass of ~50 mm^3, animals were treated intraperitoneally with OSU-HDAC-44 (7.5 mg/kg, 15 mg/kg or 30 mg/kg), TSA (1.5 mg/kg) or DMSO as control on days 1, 3, and 5 for three weeks. Prior to being sacrificed, the animals were anesthetized and blood samples were collected by intracardiac puncture for the hematological biochemistry tests. Tumor samples and mice organ tissues were resected, fixed and embedded in paraffin for histologic examination. To examine the biological effects of HDAC inhibition in tumors, mice bearing established (about 100–200 mm^3) A549 tumors were treated intraperitoneally with a single dose of OSU-HDAC-44 at 60 mg/kg. After treatment for indicated time, tumors were harvested and subjected to Western blot or immunohistochemistry analyses.

Immunohistochemistry (IHC) and fluorescence IHC assays

Tumor tissues from mice exposed to OSU-HDAC-44 were analyzed using IHC assay to detect the expression levels of cleaved caspase-3 protein and were also used for immunofluorescence and confocal microscopic analysis of Aurora B where DAPI was used to stain the DNA. The conditions were as described in the Table S2.

Statistical analysis

The SPSS program (SPSS Inc. Headquarters Chicago, Illinois) was used for all statistical analysis. Statistical analysis was performed using Student’s t-test. Data shown were representative of at least three independent experiments. Data represent mean ± SEM. P<0.05 was considered to be statistically significant.

Supporting Information

Figure S1 Effect of OSU-HDAC-44 on cell cycle progression and cell cycle-regulatory proteins. (A) The cells were treated with DMSO or 2.5 μM OSU-HDAC-44 for 12 h and then subjected to time-lapse microscopy analysis. Representative images are shown for the indicated times. Arrows pointed to the dividing cells. (B) Cells were treated with (+) or without (-) 2.5 μM OSU-HDAC-44 for 24 h or 48 h. The mean percentage of bi-nucleated cells was calculated by counting over 250 cells per experiment and plotted in the histogram. Data represent mean ± SEM from three independent experiments. *, P<0.05; **, P<0.01. (C) Cells were treated with or without 2.5 μM OSU-HDAC-44 for the indicated times and blotted for the indicated proteins. (D) Cells were treated...
with 200 ng/ml nocodazole and/or 2.5 μM OSU-HDAC-44 for 24 h, and then subjected to immunofluorescence analyses using antibodies against Aurora B (red), survivin (green), and DAPI (blue). Scale bars: 20 μm. (E) Cells were treated with 200 ng/ml nocodazole and/or 2.5 μM OSU-HDAC-44 for indicated times and blotted for the indicated proteins.

**Figure S2** OSU-HDAC-44 induced translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane. A549 and H1299 cells were treated with 2.5 μM OSU-HDAC-44 24 h, and then subjected to immunofluorescence analyses using antibody against phosphatidylserine. Scale bars: 1.0 mm.

**Figure S3** Effect of OSU-HDAC-44 on the biomarkers associated with HDAC inhibition. H1299 cells were treated with or without 2.5 μM OSU-HDAC-44 or 5 μM SAHA for the indicated times. Lysates were prepared and blotted for the indicated antibodies by Western blot analyses. The immunoblots shown are representatives of three independent experiments. Ac-H3, acetylated histone H3; Ac-H4, acetylated histone H4.

**Table S1** Inductions of histone acetylation in 33 common genes of A549 and H1299 lung cancer cells by OSU-HDAC-44.

**Table S2** The antibodies and their reaction conditions used in the present study.

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**Author Contributions**

Conceived and designed the experiments: YAT YCW. Performed the experiments: YAT WJW JWC TTW. Analyzed the data: YAT WJW JWC TTW. Contributed reagents/materials/analysis tools: SS CTC CSC. Wrote the paper: YAT YCW.

**References**

1. Dancis R, de Braud F, Fogli S, de Paas TM, Di Paolo A, et al. (2003) Pharmacogenetics of antiancer drug sensitivity in non-small cell lung cancer. Pharmacol Rev 55: 57–103.

2. Yang P, Allen MS, Aubey MC, Wampfler JA, Marks RS, et al. (2005) Clinical features of 5,628 primary lung cancer patients: experience at Mayo Clinic from 1997 to 2003. Chest 128: 452–462.

3. Pilster DG, Johanson DH, Azizoli CG, Sause W, Smith TJ, et al. (2004) American Society of Clinical Oncology treatment of unresectable non-small-cell lung cancer guideline: update 2003. J Clin Oncol 22: 330–353.

4. Sinachoome TE, Socransky MA (2009) Current treatments for advanced stage non-small cell lung cancer. Proc Am Thorac Soc 6: 233–241.

5. Sander A, Gray R, Perry MC, Brahmer J, Schiller JH, et al. (2006) Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med 355: 2542–2550.

6. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, et al. (2005) Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 353: 123–132.

7. Bhalla KN (2005) Epigenetic and chromatin modifiers as targeted therapy of hematologic malignancies. J Clin Oncol 23: 3971–3983.

8. Barlesi F, Giaccone G, Gallegos-Ruiz MI, Cordon-Cardo C, et al. (2009) Global histone modifications predict prognosis of resected non-small-cell lung cancer. J Clin Oncol 27: 4530–4540.

9. Bolden JE, Pearl MJ, Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 5: 769–784.

10. Xu WS, Parmigiani RB, Richon VM, Marks PA (2007) Histone deacetylase inhibitors: past, present and future. J Clin Cancer Res 7: 971–976.

11. Marsoni S, Damia G, Camboni G (2008) A work in progress: the clinical potential of HDAC inhibitors. Nat Rev Drug Discov 5: 769–784.

12. Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R (2007) FDA approval of HDAC inhibitors for the treatment of cutaneous T-cell lymphoma. J Biol Chem 277: 14255–14265.

13. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nature Rev Cancer 6: 38–51.

14. Lu Q, Yang YT, Chen CS, Davis M, Byrd JC, et al. (2004) Zn2+ chelating motif-tethered short-chain fatty acids as a novel class of histone deacetylase inhibitors. J Biol Chem 279: 12472–12479.

15. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors upregulate plakoglobin expression in bladder cancer cells and disrupt the epithelial-mesenchymal transition in vitro and in vivo. Int J Cancer 81: 841–848.

16. Grubbs BJ, Billmeyer BR, Austin CA, Kosakowsky M, et al. (2007) Histone deacetylase inhibitors upregulate plakoglobin expression in bladder cancer cells and disrupt the epithelial-mesenchymal transition in vitro and in vivo. Int J Cancer 81: 841–848.

17. Glotzer M (2005) The molecular requirements for cytokinesis. Science 307: 1733–1739.

18. Vesciochin B, Oudejans JJ, Vos W, Rodriguez JA, Giaccone G (2006) Frequent overexpression of aurora B kinase, a novel drug target, in non-small cell lung carcinoma patients. Mol Cancer Ther 5: 2905–2913.

19. Li J, Kolluri SK, Gu J, Dawson MI, Cao X, et al. (2000) Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. Science 298: 1159–1164.

20. Lin B, Kolluri SK, Lin F, Liu W, Han YH, et al. (2002) Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. Cell 116: 527–540.

21. Li H, Kolluri SK, Gu J, Dawson MI, Cao X, et al. (2000) Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor Nur77/TR3. Cell 116: 527–540.

22. Tang TT, Doswenko D, Jackson A, Toney L, Lewin DA, et al. (2002) The forhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J Biol Chem 277: 14253–14265.

23. Chen J, Fiskus W, Eaton K, Fernandez P, Wang Y, et al. (2009) Cotreatment with BCL-2 antagonist sensitizes cutaneous T-cell lymphoma to lethal action of HDACi-Nur77-based mechanism. Blood 113: 4038–4048.

24. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, et al. (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T44 and MDA cancer cell lines. Mol Cancer Ther 2: 151–163.

25. Mitsuishi CS, Mitsuishi NS, McMullan CJ, Poulaki V, Shringarpure R, et al. (2004) Transcriptional signature of histone deacetylation in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci U S A 101: 540–545.

26. Pearl MJ, Smyth GK, van Laar RK, Bostell DD, Richon VM, et al. (2005) Identification and functional significance of genes regulated by structurally associated proteins, including HDAC1. Proc Natl Acad Sci U S A 101: 1241–1246.
31. Li J, Lin Q, Wang W, Wade P, Wong J (2002) Specific targeting and constitutive association of histone deacetylase complexes during transcriptional repression. Genes Dev 16: 687–692.

32. Noh EJ, Jang ER, Jeong G, Lee YM, Min CK, et al. (2005) Methyl CpG-binding domain protein 3 mediates cancer-selective cytotoxicity by histone deacetylase inhibitors via differential transcriptional reprogramming in lung cancer cells. Cancer Res 65: 11400–11410.

33. Wilson AJ, Chueh AC, Togel L, Corner GA, Ahmed N, et al. (2010) Apoptotic sensitivity of colon cancer cells to histone deacetylase inhibitors is mediated by an Sp1/Sp3-activated transcriptional program involving immediate-early gene induction. Cancer Res 70: 609–620.

34. Chu YW, Yang PC, Yang SC, Shyu YC, Hendrix MJ, et al. (1997) Selection of invasive and metastatic subpopulations from a human lung adenocarcinoma cell line. Am J Respir Cell Mol Biol 17: 353–360.