Syntheses and Discovery of a Novel Class of Cinnamic Hydroxamates as Histone Deacetylase Inhibitors by Multimodality Molecular Imaging in Living Subjects

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Abstract

Histone deacetylases (HDAC) that regulate gene expression are being explored as cancer therapeutic targets. In this study, we focused on HDAC6 based on its ability to inhibit cancerous Hsp90 chaperone activities by disrupting Hsp90/p23 interactions. To identify novel HDAC6 inhibitors, we used a dual-luciferase reporter system in cell culture and living mice by bioluminescence imaging (BLI). On the basis of existing knowledge, a library of hydrazone compounds was generated for screening by coupling cinnamic hydroxamates with aldehydes and ketones. Potency and selectivity were determined by in vitro HDAC profiling assays, with further evaluation to inhibit Hsp90(α/β)/p23 interactions by BLI. In this manner, we identified compound 1A12 as a dose-dependent inhibitor of Hsp90(α/β)/p23 interactions, UKE-1 myeloid cell proliferation, p21waf1 upregulation, and acetylated histone H3 levels. 1A12 was efficacious in tumor xenografts expressing Hsp90(α)/p23 reporters relative to carrier control–treated mice as determined by BLI. Small animal 18F-FDG PET/CT imaging on the same cohort showed that 1A12 also inhibited glucose metabolism relative to control subjects. Ex vivo analyses of tumor lysates showed that 1A12 administration upregulated acetylated-H3 by approximately 3.5-fold. Taken together, our results describe the discovery and initial preclinical validation of a novel selective HDAC inhibitor. Cancer Res; 74(24); 7475–86. ©2014 AACR.

Introduction

Histone acetylation is one of the most important post-translational modifications involved in chromatin remodeling and epigenetic regulation of gene expression. In mammalian cells, histone acetylation and deacetylation are mediated by histone acetyltransferases and histone deacetylases (HDAC). In addition to histone proteins, HDACs also regulate the activities of transcription factors such as MyoD, MYC, and the estrogen receptor-α, as well as other cell circuitry proteins such as α-tubulin and Hsp90 (1). HDACs have emerged as targets for anti-cancer therapy because of the plethora of cellular processes they regulate, including cell growth, differentiation, and apoptosis (2). HDACs are classified as subfamilies class I HDAC1-3 and HDAC8; class II HDAC4-7, 9, and 10; class III (NAD+-dependent) Sirtuins, and class IV that includes only HDAC11 (1, 3, 4).

Different classes of small-molecule inhibitors have been developed to selectively or nonselectively inhibit HDACs, including suberanoylanilide hydroxamic acid (SAHA), sodium butyrate (SB), LBH589, and LAQ824. Many investigational HDACs are undergoing phase I/II clinical trials for patients with advanced cancers (5), and already two HDAC inhibitors (HDACi) have been approved for human use by the FDA (vorinostat and romidepsin), for patients with advanced cutaneous T-cell lymphoma (6–9). HDACis are also being evaluated in combination with other chemotherapy and targeted agents, including DNA-damaging agents, inhibitors of methyltransferases, topoisomerases, kinases, and the proteasome (10, 11).

The present cellular measurements of HDAC function, used in the study and development of HDACis, are largely limited to the assessment of substrate acetylation or the upregulation of target genes such p21waf1 (8, 12–17). Longitudinal kinetic studies for monitoring the efficacies of HDACi alone cannot readily be achieved without sacrificing laboratory animals at each time point before excision of tumors for ex vivo analyses.

Hsp90 is an abundant cytosolic chaperone protein that facilitates client protein folding and function.
emerged as a compelling target for therapeutic development owing to the large number of oncoprotein clients, including BCR-ABL, HER2, estrogen receptor, androgen receptor, and others (18–20). Prior research identified Hsp90 as a substrate of HDAC6 inhibition (21). Inhibition of HDAC6 by class IIa HDACi leads to hyperacetylation of Hsp90 and prevents it from interacting with p23 and its client proteins (18–20). In cell culture studies, combination of Hsp90 inhibitors (Hsp90α) and HDACis also led to enhanced inhibition of cancer growth and induction of apoptosis in some leukemia and breast cancer models in cell culture studies (4, 20), thus supports the notion of combining of Hsp90i and HDACi for cancer treatment. Beyond positive regulation through ATP binding, interactions between Hsp90(α/β) and p23 are negatively regulated by acetylation of Hsp90(α/β) (21–23). Thus, Hsp90 (α/β)/p23 interactions can be simultaneously targeted both directly (Hsp90is) and indirectly (HDACis) by combining two different classes of inhibitors. Toward this objective, we undertook to devise a noninvasive imaging strategy to monitor the function of Hsp90 as modulated by HDAC inhibition, allowing the discovery of Hsp90 acetylating novel small molecules.

We first harnessed the power of multimodality molecular imaging to evaluate the HDAC selectivity at cellular level. Using genetically encoded split Renilla luciferase (RL) reporters, we have noninvasively monitored isoform-selective interactions between Hsp90(α/β) and the co-chaperone p23 in intact 293T human kidney cancer cells in cell culture and repetitively in living mice by optical bioluminescence imaging (BLI; ref. 24). We have recently used this system for successful evaluation and validation of a novel class of Hsp90is in living mice (25). In this report, we used our Hsp90(α/β)/p23 split reporter system to indirectly monitor the efficacy of different classes of HDACi in intact cells (Fig. 1A). We have designed and synthesized a focused small-molecule HDACi library by direct coupling of a potent and HDAC-biased pharmacophore intermediate to a library of diverse aldehydes and ketones, to diversify compounds using a highly efficient chemical methodology developed previously by our groups (3). We then performed comparative dose-ranging biochemical assays to study target selectivity in a miniaturized format (Supplementary Table S1) and cellular assays for disruption of Hsp90 function using our BLI system. These efforts led to discovery of a novel HDAC inhibitor 1A12. We demonstrate the efficacy of the lead compound 1A12 in disruption of Hsp90(α/β)/p23 by BLI and its inhibition of glucose metabolism in tumor xenografts of small animals by 18F-FDG PET/CT imaging and upregulation of HDAC biomarkers by ex vivo analyses.

Materials and Methods

**Reagents and chemicals**

Cooleterazone was purchased from Nanolight Technology, dissolved in ethanol at 5 mg/mL stock. All of the animal cell culture media, FBS, the antibiotics streptomycin and penicillin (P/S), 4% to 12% gradient SDS-PAGE gels were purchased from Invitrogen. EnduRen Live Cell Substrate was purchased from Promega. 17-(Dimethylaminoethylamino)-17-demethoxy-geldanamycin (17-DMAG) and puromycin hydrochloride (100 mg/mL) were purchased from Invivogen. The purine-scaffold Hsp90i PU-H71 was synthesized and dissolved previously reported (26–28). Valproic acid sodium salt (VPA) was dissolved in distilled water as 2 mol/L stock. APHA compound 8 and trichostatin A were dissolved in DMSO (1 mg/mL). Tubacin, WT161, and MS-275 were synthesized at Broad Institute as previously described (3, 29, 30) and dissolved in DMSO as 20 mmol/L stock. All chemical reagents and solvents were purchased from Sigma-Aldrich, Acros, Novabiochem. The carrier controls that were used matched that of the highest concentration used for dilution of the compounds (100% PBS, 0.3% DMSO, and 90% β-cyclodextran: 10% DMSO for IA12, 0.01% of PBS for PU-H71; 0.1% for 17-DMAG; 0.1% for WT161; 0.1% for Tubacin; 0.1% for APHA; 0.1% MS-275) to account for their biologic effects.

**Cell culture**

Human 293T embryonic kidney cancer cells stably expressing NRL(M185V)-p23/Hsp90(α/β)-CRL (24) were grown in minimal essential medium supplemented with 10% FBS, 1% P/S solutions, and 30 µg/mL puromycin hydrochloride. NRL refers to the N-terminal portion of RL (amino acid residues 1–229) and "CRL" refers to the C-terminal portion of RL (amino acid residues 230–311). M185V refers to the mutation in NRL that led to increase in light output (31).

**Evaluation of the efficacy of HDACi alone and in combination with Hsp90i in disruption of Hsp90(α/β)/p23 interactions in intact cells in cell culture**

To determine the effect of different classes of HDACi alone and in combination with geldanamycin-based and purine-scaffold Hsp90is on Hsp90(α/β)/p23 interactions in intact cells, 3.5 × 10^4 293T and 3 × 10^4 MCF-7 cells stably expressing the split RL reporters were plated in each well in the 96-well black wall clear bottom plate (Costar) and allowed to attach for 24 hours, before treatment with different concentrations of HDACi (TSA, APHA, Valproic acid, Tubacin, and WT161) alone and in combination with geldanamycin-based (17-DMAG, 4 µmol/L) and purine-scaffold (PU-H71, 0.25 µmol/L) Hsp90is for 24 hours. Fifty microliter of EnduRen Live Cell Substrate (10 µg/mL DMSO stock diluted in complete cell culture medium) was added to each well for 1.5 hours (final concentration of 10 µmol/L), before measurement of RL activities was determined by BLI (1 minute f1 stop medium binning). Viable cell number in each well was determined by Alamar Blue assay. Complemented RL activities were normalized for viable cell numbers and to that of cells treated with carrier controls.

**Optical CCD imaging in living mice**

Animal handling was performed in accordance with Stanford University Animal Research Committee guidelines. Mice were gas anesthetized using isoflurane (2% in 100% oxygen, 1 L/min) during all procedures and kept at 37°C. Mice were imaged using a cooled CCD camera (IVIS Spectrum; PerkinElmer). Tumor establishment and BLI of 293T...
cells stably expressing Hsp90(α/β)/p23 split RL reporters and FL-eGFP [293T(α/β)-FG] cells in 7-week-old female nude mice (Charles River) were performed as described previously (25). Baseline RL activities in the implanted tumors in living mice were determined by i.v. injection of 30 μg coelenterazine (diluted in 150 μL of PBS) and image acquisition of 3 minutes at f1 stop medium binning. After a 30-minute wait for RL signals to decay, baseline FL activities were determined by i.v. injection of 163 μg of α-luciferin in 100 μL PBS with image acquisition of 10 sequences (f1 medium binning, 15 seconds) each to obtain the peak signals (average radiance). One set of mice was intraperitoneally (i. p.) injected with 50 mg/kg of 1A12 dissolved in 10% DMSO: 90% β-cyclodextran in a final volume of 150 μL (N = 4 per group). Another set of mice was treated with equal volume of 10% DMSO: 90% β-cyclodextran as a carrier control (N = 4). At different time points after treatment, follow-up RL and FL imaging was performed to monitor the effects of the 1A12 on Hsp90(α/β)/p23 interactions and viable cell numbers, respectively. Average radiance of RL was divided by that of FL signals at each time point, before normalization to that of time 0 hour for each individual mouse, and expressed as average radiance ± SEM.

Mice were euthanized after the last imaging time points, and tumors were excised and homogenized in tissue extraction buffer in the presence of Halt Complete Protease and Phosphatase Inhibitors (Pierce). The expression of p21<sup>WAF1</sup>, acetylated H3, Hsp70, and α-tubulin was determined by Western blotting.

**Data analysis**

Each experiment was repeated at least three times, and results were expressed as mean ± SEM. Statistical differences were determined by the Student t test and mixed-effect model using P < 0.05 as cutoff point. Please see Supplementary Methods for more details on determining the effect of 1A12 on Hsp90(α/β)/p23 interactions.
Results

Disruption of Hsp90(α/β)/p23 interaction by HDAC6-specific and nonselective HDACis

To determine the feasibility and specificity of using our split RL reporter system for monitoring the efficacies of different classes of HDACi in inhibition of Hsp90α/p23 and Hsp90β/p23 interactions, 293T cells stably expressing Hsp90α(β)/p23 split RL reporters were treated with nonselective HDACis TSA, HDAC1,2,3-selective MS-275 (Fig. 2A), and the HDAC6-selective inhibitor WT161 (Fig. 2B, compound structure shown in Fig. 4A) or their respective carrier controls for 24 hours. BLI of Hsp90α/p23 interactions in intact cells was performed upon addition of the RL substrate Enduren as previously described (25).

Inhibition of HDAC6 activities by class IIa and nonselective HDACi should lead to hyperacetylation of Hsp90(α/β)-CRL by histone acetyltransferase and thus reduces the complementation between NRL-p23 and Hsp90(α/β)-CRL fragments and subsequent light output (Fig. 1A). To account for the decrease in RL signals due to the decrease in viable cell numbers, RL signals (left) first were first normalized for viable cell number and then to carrier control–treated cells (right). Different pan-HDACi and HDAC6-specific inhibitors led to different levels of inhibition of Hsp90(α/β)/p23 interactions ranging from 35% to 85%, whereas the HDAC1-3–specific inhibitor MS-275 did not lead to significant inhibition of Hsp90(α/β)/p23 interactions relative to carrier control–treated cells (Fig. 1B–D). Thus, our system is specific for noninvasive monitoring of nonselective and HDAC6-selective inhibitors in intact cells.

Combinations of HDAC and Hsp90 inhibitors led to additive inhibition of Hsp90(α/β)/p23 interactions in intact cells

Hsp90is bind to the N-terminal ATP binding pocket of Hsp90 and directly disrupt Hsp90(α/β)/p23 interactions (32), whereas HDAC6-active inhibitors indirectly disrupt interactions via inhibition of Hsp90(α/β) deacetylation (33). We hypothesized that combination of Hsp90 and HDACis

Figure 2. Disruption of Hsp90(α/β)/p23 interactions by HDAC-specific inhibitors in cell culture. 293T cells stably expressing Hsp90(α/β)/p23 split RL reporters were treated with different concentrations of HDAC6-specific inhibitors, HDAC1-specific inhibitors MS-275 (A); HDAC6-specific inhibitors WT161 (B), and Tubacin, the control compound Niltubacin (C) alone or carrier control. D, to determine the additive effect of Hsp90i in combination with HDAC6 inhibitor on inhibition of Hsp90α(β)/p23 interactions, stable cells were treated with Tubacin or Niltubacin along with 250 nmol/L PU-H71 for 24 hours before determination of complemented RL activities by BLI as in Fig. 1B–D. Tubacin in combination with PU-H71 led to greater inhibition of Hsp90α/p23 interactions compared with that of Tubacin or PU-H71 alone. The control compound Niltubacin did not lead to significant inhibition of Hsp90α/p23 interactions or enhance the efficacy of PU-H71.
would lead to additive effects in inhibition of Hsp90(α/β)/p23 interactions, which can be simultaneously monitored by our split reporter complementation system. To test our hypothesis, we used the prototypical HDAC6-specific inhibitor Tubacin (29, 30), which inhibits proliferation, alters microtubules dynamics, induces apoptosis, and sensitizes cancer cells to chemotherapy agents (21, 34–38). To determine if Hsp90 inhibitors enhance the efficacy of Tubacin in disruption of Hsp90(α/β)/p23 interactions, 293T cells stably expressing the Hsp90(α/β)/p23 split reporter system were treated with Tubacin alone or in combination with PU-H71 (200 nmol/L) or 17-DMAG (1 μmol/L). BLI was performed 24 hours after treatment as before. Niltubacin, the inactive analogue of Tubacin, was used as a negative control.

As shown in Fig. 2C and D, Tubacin in combination with PU-H71 led to greater disruption of Hsp90(α/β)/p23 interaction, as shown by the further reduction in bioluminescence signals relative to cells treated with Tubacin alone. On the other hand, the control compound Niltubacin had no effect on Hsp90(α/β)/p23 interaction alone and did not enhance the efficacy of PU-H71 at the doses tested, relative to carrier control–treated cells (Supplementary Fig. S1A). Similar results were seen with another Hsp90i 17-DMAG in 293T cells (Supplementary Figs. S1B and S2) and in MCF-7 cells that express the Hsp90(α/β)/p23 split RL reporters (data not shown).

To confirm that the decrease in BLI (Figs. 1 and 2) correlates with the inhibition of HDAC6, the expression of p21Vaf1, acetylated histone H3 (ac-H3), and acetylated α-tubulin (standard biomarkers of HDAC6 inhibition) in cells treated with different HDACis was determined by Western blotting (Fig. 3). Normalization to α-tubulin shows that the expression of acetylated α-tubulin was upregulated in cells treated with non-selective HDAC and HDAC6-specific inhibitors, but not in cells treated with the HDAC1-3 inhibitor MS-275, Niltubacin, or carrier controls. Treatment with HDACis also led to increased acetylation of H3, as expected. Thus, our reporter system was specific for monitoring inhibition of HDAC6 activities in intact cells through indirect imaging of Hsp90(α/β)/p23 interaction.

Discovery of novel HDAC6 inhibitors through indirect monitoring of Hsp90(α/β)/p23 interactions in intact cells

In our previous study, we developed a convergent synthetic platform to establish a hydrazone library that enabled us to identify the selective HDACis, provide structure activity relationship (SAR), and discovered a pan-HDAC inhibitor panobinostat (3). Encouraged by this discovery, we further coupled para- and meta-cinnamic hydroxamates that are HDAC recognition-binding warheads with more than 384 commercially available aldehydes (Supplementary Fig. S3A) and ketones using the hydrazone formation chemistry (Fig. 4A) by coupling hydrazide directly to aldehydes and ketone in DMSO in a 96-well plate format, and generated a focused library of cinnamic hydroxamates with the structure diversity on the hydrazone end to explore the effect of cap space on the inhibition of HDAC activities (Fig. 4A). All compounds were profiled with an in vitro fluorogenic HDAC assays (3) using purified proteins (HDACs 1–9) to confirm the activities and selectivity against HDACs 1–9 (Supplementary Fig. S3B and S3C and Supplementary Table S1). The 384 compounds were further evaluated for their ability to disrupt Hsp90 (α/β)/p23 interactions in intact cells. 293T cells stably expressing Hsp900c/p23 or Hsp90β/p23 split RL reporters were treated with each of 384 compounds at 4.5 μmol/L or carrier control for 24 hours, before RL imaging as before. Cells treated with WT161 (10 μmol/L) and PU-H71 (5 μmol/L) served as positive controls for HDAC and Hsp90 inhibition, respectively.

Figure 4B shows various degrees of inhibition of RL signals by 72 of the 384 compounds (rows A–F; columns 1–12) on Hsp900c/p23 interactions, relative to carrier control–treated cells. WT161, a HDAC6-selective inhibitor (Fig. 4A), and PU-H71 both led to decrease in RL signals, as expected. Similar results were seen for 293T cells stably expressing Hsp90β/p23 interactions in intact cells. 293T cells stably expressing Hsp90βc/p23 or Hsp90β/p23 split RL reporters were treated with each of 384 compounds at 4.5 μmol/L or carrier control for 24 hours, before RL imaging as before. Out of the 384 compounds we synthesized and tested, three compounds 2H9, 2C2, and 1A12 (Fig. 5A) led to the highest level of inhibition of Hsp90(α/β)/p23 interaction (>42%). To compare the efficacy of these three compounds with that of WT161, all three hits were re-synthesized and tested with 293T cells expressing the split RL reporters for 24 hours treatment (Fig. 5B). RL signals were normalized for viable cell number in each well. Figure 5C shows that 1A12 was more effective than 2C2 and 2H9 in inhibition of Hsp900c/p23 interactions (left). 1A12, 2C2, and WT161 led to similar inhibition of Hsp90βc/p23 interactions (right). The efficacy of 1A12 was also similar to that of WT161 in disruption of both Hsp90α/β/p23 interactions, whereas 2H9 was the least effective compound. Our results indicate that 1A12 is most effective against HDAC1 (IC50 = 0.2 nmol/L), followed by HDAC3 (IC50 = 1.4 nmol/L) and HDAC6 (IC50 = 1.7 nmol/L). 1A12 also inhibits HDACs 2, 4,

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Specificity of using dual-reporter system for monitoring the efficacy of HDACis. To determine the specificity of using the dual-reporter system for noninvasive monitoring of the efficacies of HDACis, 293T cells were treated with the indicated HDACis for 24 hours before Western blotting of the expression of p21Vaf1, Hsp70, ac-H3, and acetylated α-tubulin. The blots were stripped and reprobed with α-tubulin antibody to control for protein loading.
and 9 at higher concentrations. Furthermore, all of the HDAC inhibitors appeared to be more selective for inhibition of Hsp90α/p23 interactions (Fig. 5C, left), relative to Hsp90β/p23 interactions (right).

To validate the mechanisms of lead compounds in inhibition of HDAC activities, 293T cells expressing Hsp90 (α/β)/p23 split RL reporters were treated with different concentrations of 2H9, 1A12, and 2C2 or carrier control for 24 hours. WT161 (30 μmol/L) and PU-H71 (5 μmol/L) served as positive controls for inhibition of HDAC6 and Hsp90 activities, respectively. The expression of p21waf1, acet-H3, and Hsp70 was determined by Western blotting. Figure 5D shows 2H9, 1A12, and 2C2 led to dose-dependent increases in expression of p21waf1, acet-H3, and Hsp70 in 293T cells stably expressing the Hsp90α/p23 sensor, relative to carrier control–treated cells. WT161 also led to upregulation of p21waf1 and acet-H3, followed by 2C2 and 2H9.

To further determine if the observed effects of 1A12 on inhibition of Hsp90(α/β)/p23 interactions and glucose metabolism was due to inhibition of HDAC activities, we synthesized a methyl ester analogue of 1A12 (Methyl 3-(4-((E)-2-((Z)-2-bromo-3-phenylallylidene)hydrazinecarbonyl)phenyl)propanoate, abbreviated as 1A12-ME). It is well known that hydroxamate is the binding head for HDAC (39, 40); therefore, we expected that 1A12-ME with the methyl ester will not bind to HDAC6 or inhibits its activity due to lack of a double bond in the COHOOH position.

To determine the specificity of 1A12-ME on inhibition of HDAC6, as well as HDAC1 and HDAC3, in vitro deacetylases assays were performed. Compared with SAHA, 1A12-ME had no effect on HDAC1, 3, and 6 activities except at the highest concentration tested (5 and 10 μmol/L; Supplementary Table S1). 1A12-ME also did not lead to upregulation of HDAC biomarkers (α/β)/p23 sensor. In agreement with the RL imaging of Hsp90(α/β)/p23 interactions (Fig. 5C), 1A12 was the most potent compound in upregulation of the expression of p21waf1 and acet-H3, followed by 2C2 and 2H9.
Characterization of the lead HDACis. A, chemical structures of the lead compounds 1A12, 2C2, and 2H9. B, dose-dependent inhibition of Hsp90α/p23 interactions by the lead compounds. 293T cells stably expressing the Hsp90α/p23 sensors were treated with different concentrations of the indicated compounds for 24 hours before RL imaging. WT161 was used as a positive control. C, net effect of 1A12 (squares), 2C2 (triangles), and 2H9 (diamonds) on Hsp90α/p23 interactions, compared with that of 1A12 (46% and 19% at 30 μmol/L and 7.5 μmol/L, respectively), compared with that of 1A12 (46% and 44% at 30 μmol/L). 1A12-ME also inhibits proliferation of UKE-1 myeloplastic proliferative cells, alone or in combination with doxorubicin or the HDACi PU-H71 (Supplementary Fig. S7). Thus, our assay allows for the rapid screening of novel HDACis through indirect imaging of Hsp90α/p23 interactions in intact cells.

Noninvasive monitoring of the efficacy of 1A12 in disruption of Hsp90α/p23 interactions in mice

The cellular imaging and biochemical evaluation established 1A12 as the most potent compound within the
384-compound library. To noninvasively monitor the efficacy of the compound in HDAC inhibition and viable cell numbers in living mice, we introduced a second reporter (FL-eGFP) into the 293T cell stably expressing Hsp90 (α/β)/p23 sensors as previously described (25). FL was used to determine viable cell number, which increases as the cells proliferate and decreases when the cells die upon treatment with HDACis. The efficacy of 1A12 in disruption of Hsp90(α/β)/p23 interactions was monitored by RL imaging upon tail-vein injection of coelentarazine. The effects of these compounds on viable cell number were subsequently monitored by FL imaging upon tail-vein injection of d-luciferin, because the two substrates do not cross-react (41). We have previously determined that 50 mg/kg 1A12 was insufficient for upregulation of the acetylated tubulin in tumor xenografts (data not shown). Baseline RL and FL signals were determined by sequential BLI imaging before treatment with 80 mg/kg of 1A12 in 10% DMSO:90% β-cyclodextran or equal volume of carrier control (10% DMSO:90% β-cyclodextran) via i.p. injections for two days after imaging (four doses total). Mice were reimaged for RL and FL signals at 15 hours after the first dose, and 41 hours after two more doses of treatment.

Figure 6A shows the RL signals (i.e., Hsp90(α/β)/p23 interactions) in mice decreased at 15 hours after 1A12 treatment, but increased in mice treated with the same volume of carrier control (top left). To determine the effect of 1A12 on viable cell number, FL imaging was performed after RL imaging at each time point. To account for effect of viable cell numbers by 1A12 on Hsp90(α/β)/p23 interactions, we normalized the RL signals to that of FL signals for each time point, and to time 0 hour for each mouse. A mixed-effects model was used to account for random variation between mice in these repeated measure experiments (see Supplementary Methods). Figure 6B shows that 1A12 led to net decrease in Hsp90α/p23 interactions (RL/FL ratios) in...
293T tumor xenografts expressing Hsp90α/p23 split RL reporters at 15 and 40 hours (P < 0.014 relative to carrier control–treated mice). 1A12 did not significantly alter Hsp90α/p23 interactions in tumor xenografts (P > 0.05). Our analyses also indicate there was no significant difference between the 15- and 40-hour time points in either the Hsp90α/p23 or Hsp90β/p23 groups (P > 0.05).

Monitoring downstream effects of 1A12 by 18F-FDG uptake by small animal PET imaging

In addition to determining the efficacy of 1A12 in disruption of Hsp90(α/β)/p23 interactions by BLI (Fig. 6), we also monitored its downstream effects on glucose metabolism in the same cohort of living mice by small animal 18F-FDG PET/CT imaging. 18F-FDG PET/CT has been routinely used for repetitive and noninvasive monitoring of chemotherapy responses both in small animals and in human. The short radioactive half-life (110 minutes) of 18F (42) also permits repetitive imaging for before and after 1A12 treatment. Baseline 18F-FDG uptake in tumor-bearing mice was determined before and after 1A12 treatment. The percentage mean ID/g tissue of 18F-FDG uptake was calculated using the OSEM2D method upon normalization of injected dose (43), and the percentage change at day 2 relative to day 0 was determined for each tumor site.

In carrier control–treated mice, relative to time 0 hour, the mean increase in percent ID/g of 18F-FDG uptake in 293T tumors expressing Hsp90(α/β)/p23 RL reporters (N = 10) was 181 ± 37% (Fig. 7B). On the other hand, mean change in percent ID/g of 18F-FDG uptake in 1A12-treated tumors (N = 8) decreased by 64 ± 23% (P < 0.01 relative to carrier control–treated mice). Therefore, in addition to inhibition of Hsp90(α/β)/p23 interactions, 1A12 also inhibits glucose metabolism in tumor xenografts in living mice. To confirm the efficacy of 1A12 and 2C2 as an HDAC inhibitor in mice, tumors were excised from the mice after the last imaging time point, and the expression of p21waf1 and act-H3 was determined by Western blotting. Quantitation of the images shows that 1A12 led to upregulation of act-H3 by about 3.5-fold in 293T xenografts (P < 0.05) but did not significantly change the level of p21waf1 (P > 0.05; Supplementary Fig. S8). Our results confirm the efficacy of 1A12 as a HDACi, both in cell culture and in living mice.

Discussion

Hydroxamic acids have been used as HDACs inhibitors in a variety of compounds due to their ability to bind to zinc within the catalytic pocket of HDACs. Here, we have further investigated the potent cinnamic hydroxamate pharmacophore that previously led to our discovery of the first truly nonselective HDACi (pandacostat; ref. 3), with an objective of identifying cell-permeable HDACis capable of disrupting Hsp90 function. Encouraged by these exciting findings, the library has been further expanded by condensation of para- and meta-hydrazide with variety of aldehydes or ketones (Fig. 4A). The resulted library was evaluated by enzymatic assay as well as cellular assay. The lead compounds were scaled up for in vivo evaluation.

In the current work, we successfully incorporated multi-modality molecular imaging into a discovery platform to identify novel cinnamic hydroxamates-based HDACis. We accomplished our goals of monitoring HDAC6 activities via BLI of Hsp90(α/β)/p23 interactions in intact cells. Upon validating the sensitivity and specificity of our system with known HDACi, we focused our efforts on screening our custom-synthesized compound library and discovered that the meta-cinnamic hydroxamate derivatives, such as the lead compound 1A12, have shown efficacy in disruption of Hsp90α/p23 interactions. We have also noninvasively and repetitively monitored the inhibitory effects of 1A12 in 293T tumor xenografts in living mice on Hsp90α/p23 interactions by BLI (Fig. 6) and glucose metabolism by 18F(FDG) PET/CT (Fig. 7) and validated its mechanism as an HDACi by ex vivo analyses (Supplementary Fig. S8).
Because the HDAC I selective inhibitors, such as MS275, did not provide the same effect on inhibition of Hsp90 (α/β)/p23 interactions (Figs. 2A and 3), we proposed the effects on inhibition of glucose metabolism (Fig. 7) we observed in tumor xenografts in mice were in part due to HDAC6 inhibition by 1A12.

**Specificity of 1A12 as a novel hydroxamate-based HDACi**

We confirmed HDAC6 as one of the targets of 1A12 and examined its efficacy as an HDACi in cell culture. 1A12 led to dose-dependent decreases in Hsp90(α/β)/p23 interactions with corresponding increases in the levels standard HDAC biomarkers (Fig. 5). It was more selective for inhibition of BLI signals from Hsp90α/p23 interactions, relative to Hsp90α/p23 interactions in a dose-dependent manner. We have performed short-term experiments to determine if the lead HDACi (1A12) alters FL activities and protein expression, using the 293T cells that stably express full-length FL (293T/FL). 293T/FL cells were exposed to 1A12 (30 μmol/L) in different sequences as follow: (i) 1A12 for 1 to 15 minutes before addition of β-luciferin; (ii) β-luciferin for 5 minutes before addition of 1A12; or (iii) simultaneous addition of β-luciferin with 1A12. We did not observe any significant effect of 1A12 on FL activities in 293T/FL cells regardless of the sequence of addition (Supplementary Fig. S6C and data not shown). Similar results were observed in 293T/α-FG and 293T/β-FG cells (data not shown). To determine the effect of 1A12 on expression of FL protein, 293T/FL cells were exposed to 1A12 (30 μmol/L) for 1 to 15 minutes or for 24 hours at 7.5 to 30 μmol/L before Western blotting. 1A12 treatment did not significantly alter the expression of FL protein at all the time points and concentrations tested (Supplementary Fig. S6D). In sum, our results indicated that 1A12 could be used as a second reporter to account for the change in viable cell number due to drug treatment. Using an inactive analogue of 1A12 (1A12-ME), we have further confirmed that the biologic effects of 1A12 were in part due to inhibition of HDAC6 (Supplementary Figs. S5 and S6).

**A unified dual split RL and FL reporter system accelerated drug discovery, mechanism validation, and lead optimization in living subjects**

Our split reporter assay (Fig. 1A) allows us to specifically monitor the interactions between Hsp90(α/β) and p23 in intact cells, which is negatively regulated by HDAC6. Even though HDAC6-selective and pan-HDACis can both lead to disruption of Hsp90(α/β)/p23 interactions, we can monitor its effect alone and in combination with other Hsp90is on HDAC6 noninvasively in intact cells. As described in Fig. 2C and D and Supplementary Fig. S2, we were able to monitor the combined effect of Hsp90 inhibitors (PU-H71 and 17AAG) with HDACis (Tubacin and WT161) on inhibition of Hsp90(α/β)/p23 interactions. Given the upregulation of Hsp90 activities is specific for cancer cells, the development of HDAC6-selective inhibitors could lead to more effective combinatorial therapies aimed at targeting both HDAC6 (directly) and Hsp90 (indirectly). It may also possibly reduce toxicities and off-target effects associated with pan-HDACis.

We used our Hsp90(α/β)/p23 split RL reporter system (25) for screening of novel HDACis in cell culture and subsequently in living mice. Our strategy will allow rapid evaluation of different structural analogues generated by medicinal chemistry with better potency and bioavailability. The leads will then be tested in mice and this will significantly reduce the costs of scaling up the syntheses of compounds. Furthermore, the reporters can be introduced into specific cancer cell lines that overexpress HDAC6 (21).

**Importance of multimodality molecular imaging for human clinical translation**

Our multimodality molecular imaging platform represents a significant advancement in accelerating drug development. The ease, relative low cost, noninvasive and high sensitivity of BLI allow rapid screening of large chemical libraries in very small quantities and subsequent SAR studies. Currently, human PET/CT imaging has been used to monitor tumor progression, evaluation, and response to therapy (44–46). PET imaging has also been used for preclinical studies for evaluation of the efficacy of different HDACi in small animal models. For example, the HDAC inhibitor SAHA was radiolabeled with 18F for PET/CT imaging of ovarian carcinomas treated with SAHA (47), whereas 18F-FLT PET has been used to monitor the effect of LAQ824 and PXD101 on cell proliferation in cell in carcinomas (48, 49). Using the same cohort of mice for BLI imaging, we also monitored the inhibition of glucose metabolism by 1A12 using 18F-FDG (Fig. 7). A multimodality imaging approach will therefore provide specific mechanistic and temporal information pertaining to drug treatment and identify determinants for response to therapy.

In summary, we have discovered and validated a novel class of hydroxamate-based HDACis by coupling multimodality molecular imaging to biased chemical library synthesis and iterative biochemical profiling. Our workflow allows rapid identification of cell-permeable lead compounds that can be validated in cell culture followed by monitoring of *in vivo* efficacy and downstream effects in living mice. The results of this study will significantly accelerate the development of next generations of therapeutics aimed at inhibiting specific HDACs.

**Disclosure of Potential Conflicts of Interest**

R. Mazitschek is a consultant/advisory board member for Shape Pharmaceuticals and Acetylon Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C.T. Chan, J. Qi, W. Smith, N. West, J.E. Bradner

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21. R. Mazitschek
Syntheses and Discovery of a Novel Class of Cinnamic Hydroxamates as Histone Deacetylase Inhibitors by Multimodality Molecular Imaging in Living Subjects


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