Syntheses and Discovery of a Novel Class of Cinnamic Hydroxamates as Histone Deacetylase (HDAC) 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 employed a dual-luciferase reporter system in cell culture and living mice by bioluminescence imaging (BLI). Based on 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/β)/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 ~3.5-fold. Taken together our results describe the discovery and initial preclinical validation of a novel selective HDAC inhibitor [191 words].
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 (HATs) and histone deacetylases (HDACs). 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 non-selectively 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 have been approved for human use by the FDA (Vorinostat® and Romidespin®), for patients with advanced cutaneous T-cell lymphoma (6-9). HDAC inhibitors are also being evaluated in combination with other chemotherapy and targeted agents, including DNA-damaging agents, inhibitors of methylthransferases, topoisomerases, kinases and the proteasome (10, 11).

The present cellular measurements of HDAC function, used in the study and development of HDAC inhibitors, are largely limited to the assessment of substrate acetylation or the upregulation of target genes such p21 (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 prior to excision of tumors for ex-vivo analyses.

Hsp90 is an abundant cytosolic chaperone protein that facilitates client protein folding and function. Hsp90 has emerged as a compelling target for therapeutic development owing to the large number of oncoprotein clients, including BCR-ABL, HER2, ER, AR and others (18-20). Prior research identified Hsp90 as a substrate of HDAC6 inhibition (21). Inhibition of HDAC6 by class Ila 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 and HDAC inhibitors 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 (Hsp90 inhibitors) and indirectly (HDAC inhibitors) by combining two different classes of inhibitors. Toward this objective, we undertook to devise a non-invasive 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 non-invasively 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) (24). We
have recently used this system for successful evaluation and validation of a novel class of Hsp90 inhibitors in living mice (25). In this report, we utilized our Hsp90(α/β)/p23 split reporter system to indirectly monitor the efficacy of different classes of HDACi in intact cells (Figure 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 (Supplemental Table 1) 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 up-regulation of HDAC biomarkers by ex-vivo analyses.
Materials and Methods

Reagents and Chemicals. Coelenterazine was purchased from Nanolight technology (Pinetop, AZ), dissolved in ethanol as 5mg/ml stock. All of the animal cell culture media, fetal bovine serum (FBS), the antibiotics streptomycin and penicillin (P/S), 4-12% gradient SDS-PAGE gels were purchased from Invitrogen (Carlsbad, CA). EnduRen Live Cell Substrate™ was purchased from Promega (Madison, WI). 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) and puromycin hydrochloride (100 mg/ml) were purchased from Invivogen (San Diego, CA). The purine-scaffold Hsp90 inhibitor PU-H71 was synthesized and dissolved previously reported (26-28). Vaproic acid sodium salt (VPA) was dissolved in distilled water as 2 M 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 [37] and dissolved in DMSO as 20 mM 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 1A12, 0.01% of PBS for PU-H71; 0.1% for 17-DMAG; 0.1% for WT161; 0.1% for Tubacin; 0.1% for APHA8; 0.1% MS275) to account for their biological 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 (MEM) 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 (29).
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 Hsp90 inhibitors on Hsp90(α/β)/p23 interactions in intact cells, $3.5 \times 10^4$ 293T and $3 \times 10^4$ MCF-7 cells stably expressing the split RL reporters were plated in each well in the 96-well black wall clear bottom plate (Costar, Corning, NY) and allowed to attach for 24 hours, prior to treatment with different concentrations of HDACi (TSA, APHA8, Valproic acid, Tubacin and WT161) alone and in combination with geldanamycin-based (17-DMAG, 4μM) and purine-scaffold (PU-H71, 0.25 M) Hsp90 inhibitors for 24 hours. 50 μl of EnduRen Live Cell Substrate™ (10 g/ml DMSO stock diluted in complete cell culture medium) was added to each well for 1.5 hours (final concentration of 10 μM), prior to measurement of RL activities were determined by BLI (1 min 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 isofluorane (2% in 100% oxygen, 1L/min) during all procedures and kept at 37°C. Mice were imaged using a cooled CCD camera (IVIS Spectrum, Perkin Elmer, MA). 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) was performed as described previously (25). Baseline RL activities in the implanted tumors in living mice were determined by i.v. injection of 30 μg cltz (diluted in 150 μl of PBS) and image acquisition of 3 mins at f1 stop medium binning. After a 30 mins wait for RL signals to
decay, baseline FL activities were determined by i.v. injection of 163 μg of D-Luc 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 post-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, prior to normalization to that of time 0 hr for each individual mouse, and expressed as average radiance ± S.E.M.

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, IL). The expression of p21^{waf1}, acetylated H3, Hsp70 and α-tubulin were determined by western blotting.

**Data analysis.** Each experiment was repeated at least three times and results were expressed as mean +/- standard error of means (S.E.M.). Statistical differences were determined by student t-test and mixed-effect model using p < 0.05 as cut-off point. Please see supplemental 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 non-selective-HDAC inhibitors. 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 Hsp90(α/β)/p23 split RL reporters were treated with non-selective-HDAC inhibitors TSA; HDAC1,2,3-selective MS-275 (Figure 2A) and the HDAC6-selective inhibitor WT161 (Figure 2B, compound structure shown in Figure 4A) or their respective carrier controls for 24 hours. BLI of Hsp90(α/β)/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 non-selective HDACi should lead to hyperacetylation of Hsp90(α/β) -CRL by HAT and thus reduces the complementation between NRL-p23 and Hsp90(α/β)-CRL fragments and subsequent light output (Figure 1A). To account for the decrease in RL signals due to the decrease in viable cell numbers, RL signals (left panels) first were first normalized for viable cell number and then to carrier control-treated cells (right panels). Different pan-HDACi and HDAC6-specific inhibitors led to different levels of inhibition of Hsp90(α/β)/p23 interactions ranging from 35-85%, while 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 (Figures 1B-D). Thus our system is specific for non-invasive monitoring of non-selective and HDAC6-selective inhibitors in intact cells.

Combinations of histone deacetylase and Hsp90 inhibitors led to additive inhibition of Hsp90(α/β)/p23 interactions in intact cells. Hsp90 inhibitors bind to the N-terminal ATP binding pocket of Hsp90 and directly disrupt Hsp90(α/β)/p23 interactions (30), while HDAC6-
active inhibitors indirectly disrupt interactions via inhibition of Hsp90(α/β) deacetylation (31).

We hypothesized that combination of Hsp90 and HDAC inhibitors 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 utilized the prototypical HDAC6-specific inhibitor Tubacin (32, 33), 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 nM) or 17-DMAG (1 μM). BLI was performed 24 hours post-treatment as before. Niltubacin, the inactive analogue of Tubacin, was used as a negative control.

As shown in Figures 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 (Supplemental 1A). Similar results were seen with another Hsp90 inhibitor 17-DMAG in 293T cells (Supplemental Figures 1B and 2) 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 (Figures 1 and 2) correlates with the inhibition of HDAC6, the expression of p21\textsuperscript{waf1}, acetylated histone H3 and acetylated α-tubulin (standard biomarkers of HDAC6 inhibition) in cells treated with different HDAC
inhibitors were determined by western blotting (Figure 3). Normalization to α-tubulin shows that the expression of acetylated α-tubulin was up-regulated 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 HDAC inhibitors 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 HDAC inhibitors, provide structure-activity relationship (SAR), and discovered a pan-HDAC inhibitor pandacostat (3). Encouraged by this discovery, we further coupled para- and meta-cinnamic hydroxamates that are HDAC recognition biding warhead with more than 384 commercially available aldehydes (Supplemental Figure S3A) and ketones utilizing the hydrazone formation chemistry (Figure 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 (Figure 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 HDAC 1-9 (Supplemental Figures S3B, S3C and Supplemental Table 1). The 384 compounds were further evaluated for their ability to disrupt Hsp90(α/β)/p23 interactions in intact cells. 293T cells stably expressing Hsp90α/p23 or Hsp90β/p23 split RL reporters were treated with each of 384 compounds at 4.5 μM or carrier control for 24 hours, prior to RL imaging as before. Cells treated with WT161 (10 μM) and PU-H71 (5
μM) served as positive controls for HDAC and Hsp90 inhibition, respectively.

Figure 4B shows that various degrees of inhibition of RL signals by 72 of the 384 compounds (rows A-F, columns 1-12) on Hsp90α/p23 interactions, relative to carrier control treated cells. WT161, a HDAC6 selective inhibitor (Figure 4A) and PU-H71 both led to decrease in RL signals, as expected. Similar results were seen for 293T cells stably expressing Hsp90β/p23 split RL reporters (data not shown). RL signals in each well were normalized for viable cell number and to carrier control. Out of the 384 compounds we synthesized and tested, three compounds 2H9, 2C2 and 1A12 (Figure 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 (Figure 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 Hsp90α/p23 interactions (left panel). 1A12, 2C2 and WT161 led to similar inhibition of Hsp90β/p23 interactions (right panel). The efficacy of 1A12 was also similar to that of WT161 in disruption of both Hsp90(α/β)/p23 interactions, while 2H9 was the least effective compound. Our results indicate that 1A12 is most effective against HDAC1 (IC50 = 0.2 nM), followed by HDAC3 (IC50 = 1.4 nM) and HDAC6 (IC50 = 1.7nM). 1A12 also inhibits HDACs 2, 4, 5 and 9 at higher concentrations. Furthermore, all of the HDAC inhibitors appeared to be more selective for inhibition of Hsp90α/p23 interactions (Figure 5C, left), relative to Hsp90β/p23 interactions (right panel).

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 μM) and PU-H71 (5 μM) served as positive controls for inhibition of HDAC6 and Hsp90 activities, respectively. The expression of p21waf1, acetylated histone H3 (acet-H3) and Hsp70 were
determined by western blotting. Figure 5D shows 2H9, 1A12 and 2C2 led to dose-dependent increases in expression of p21waft, acet-H3 and Hsp70 in 293T cells stably expressing the Hsp90α/p23 sensor, relative to carrier control treated cells. WT161 also led to up-regulation of p21 and acet-H3. PU-H71 led to increased expression of Hsp70 and p21waft as expected [35], without affecting the levels of Acet-H3. Normalization to α-tubulin shows that 2C2, 1A12, 2H9 and WT161 led to increased expression of acet-H3 and p21waft (Suppl. Figure S4). Similar results were observed for that of 293T cells stably expressing Hsp90/p23 sensor. In agreement with the RL imaging of Hsp90(α/β)/p23 interactions (Figure 5C), 1A12 was the most potent compound in up-regulation of the expression of p21waft and act-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 1A12-ME with the methyl ester will not bind to HDAC6 or inhibits its activity due to lack of a double bond in the COHOH 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 to SAHA, 1A12-ME had no effect on HDAC1, 3 and 6 activities except at the highest concentration tested (5 and 10 μM) (Supplementary Figure S5A). On the other hand, the IC50 for 1A12 for inhibition of HDAC6 was 1.7 nM (Supplemental Table 1). 1A12-ME also did not lead to upregulation of HDAC biomarkers (acetylated histone H3, Acet-H3) in 293T cells stably expressing Hsp90(α/β)/p23 split RL reporters, compared to those treated with 1A12 (Figure S5B). To further confirm the specificity
of 1A12 for inhibition of HDAC6 activity, 293T cells stably expressing Hsp90(α/β)/p23 split RL reporters were treated with different concentrations of 1A12 or 1A12-ME or the carrier control for 24 hours, prior to bioluminescence imaging (BLI) of Hsp90(α/β)/p23 interactions (RL imaging) and viable cell number (FL imaging) (Figure S6A and data not shown). 1A12-ME had minimal effect on Hsp90(α/β)/p23 interactions (16% and 19% at 30 μM for Hsp90α/p23 and Hsp90β/p23 interactions, respectively), compared to that of 1A12 (46% and 44% at 30 μM for Hsp90α/p23 and Hsp90β/p23 interactions, respectively)(Supplemental Figure S6B). Thus the activities of 1A12 reported on inhibition of Hsp90(α/β)/p23 interactions and subsequent glucose metabolism is specific for its ability to inhibit HDAC6.

1A12 also inhibits proliferation of UKE-1 myeloplastic proliferative cells, alone or in combination with Doxorubicin or the Hsp90 inhibitor PU-H71 (Supplementary Figure S7). Thus our assay allows for the rapid screening of novel HDAC inhibitors through indirect imaging of Hsp90(α/β)/p23 interactions in intact cells.

**Non-invasive monitoring of the efficacy of 1A12 in disruption of Hsp90(α/β)/p23 interactions in mice.** The cellular imaging and biochemical evaluation established 1A12 as the most potent compound within the 384-compound library. To non-invasively 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 cell proliferate and decrease when the cells die upon treatment with HDAC inhibitors. 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, since 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 prior to 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 post imaging (4 doses total). Mice were re-imaged for RL and FL signals at 15 hours after the first dose, and 41 hours post two more doses of treatment.

Figure 6A show the RL signals (i.e. Hsp90(α/β)/p23 interactions) in mice decreased at 15 hours post 1A12 treatment, but increased in mice treated with the same volume of carrier control (top left panel). 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 hr for each mouse. A mixed effects model was used to account for random variation between mice in these repeated measure experiments (see Supplemental Method). 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 Hsp90β/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 $^{18}$F-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 (Figure 6), we also monitored its downstream effects on glucose
metabolism in the same cohort of living mice by small animal $^{18}$F-FDG PET/CT imaging. $^{18}$F-FDG PET/CT has been routinely used for repetitive and non-invasive monitoring of chemotherapy responses both in small animals and in human. The short radioactive half-life (110 mins) of $^{18}$F (42) also permits repetitive imaging for pre- and post 1A12 treatment. Baseline $^{18}$F-FDG uptake in tumor bearing mice was determined pre- and post 1A12 treatment. The %mean ID/g tissue of $^{18}$F-FDG uptake was calculated using the OSEM2D method upon normalization of injected dose (43), and the % change at day 2 relative to day 0 was determined for each tumor site.

In carrier control treated mice, relative to time 0 hr, the mean increase in %ID/g of $^{18}$F-FDG uptake in 293T tumors expressing Hsp90(α/β)/p23 RL reporters (N=10) was 181 ± 37% (Figure 7B). On the other hand, mean change in %ID/g of $^{18}$F-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 p21$^{\text{waf1}}$ and act-H3 was determined by western blotting. Quantitation of the images shows that 1A12 led to up-regulation of acet-H3 by about 3.5-fold in 293T xenografts ($p < 0.05$) but did not significantly change the level of p21$^{\text{waf-1}}$ ($p > 0.05$) (Supplemental Figure S8). Our results confirm the efficacy of 1A12 as HDAC inhibitors, both in cell culture and in living mice.
Discussion

Hydroxamic acids have been utilized 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 which previously led to our discovery of the first truly non-selective HDAC inhibitor (pandacostat (3)), with an objective of identifying cell-permeable HDAC inhibitors 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 (Figure 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 multimodality molecular imaging into a discovery platform to identify novel cinnamic hydroxamates-based HDAC inhibitors. 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 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 Hsp90(α/p23 interactions. We have also non-invasively and repetitively monitored the inhibitory effects of 1A12 in 293T tumor xenografts in living mice on Hsp90α/p23 interactions by BLI (Figure 6) and glucose metabolism by 18F[FDG] PET/CT (Figure 7) and validated its mechanism as an HDAC inhibitor by ex-vivo analyses (Supplemental Figure 8). Since the HDAC I selective inhibitors, such as MS275 did not provide the same effect on inhibition of Hsp90(α/β)/p23 interactions (Figures 2A and 3), we proposed the effects on inhibition of glucose metabolism (Figure 7) we observed in tumor xenografts in mice was 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 HDAC inhibitor in cell culture. 1A12 led to dose-dependent decreases in Hsp90(α/β)/p23 interactions with corresponding increases in the levels standard HDAC biomarkers (Figure 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 HDAC inhibitor (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 μM) in different sequences as follow: a) 1A12 for 1-15 mins prior to addition of D-Luc; b) D-Luc for 5 mins prior to addition of 1A12; or c) simultaneous addition of D-Luc with 1A12. We did not observe any significant effect of 1A12 on FL activities in 293T/FL cells regardless of the sequence of addition (Supplemental Figure 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 μM) for 1-15 mins or for 24 hours at 7.5-30 μM prior to western blotting. 1A12 treatment did not significantly alter the expression of FL protein at all the time points and concentrations tested (Supplemental Figure 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 the biological effects of 1A12 were in part due to inhibition of HDAC6 (Supplemental Figures 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 (Figure 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-HDAC inhibitors can both lead to disruption of Hsp90(α/β)/p23 interactions, we can monitor its
effect alone and in combination with other Hsp90 inhibitors on HDAC6 non-invasively in intact cells. As described in (Figures 2C and D and Supplemental Figure S2), we were able to monitor the combined effect of Hsp90 inhibitors (PU-H71 and 17AAG) with HDAC inhibitors (Tubacin and WT161) on inhibition of Hsp90(α/β)/p23 interactions. Given the up-regulation 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-HDAC inhibitors.

We utilized our Hsp90(α/β)/p23 split RL reporter system (25) for screening of novel HDAC inhibitors 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 over-express HDAC6 (21).

**Importance of multimodality molecular imaging for human clinical translation.** Our multi-modality molecular imaging platform represents a significant advancement in accelerating drug development. The ease, relative low-cost, non-invasive and high sensitivity of BLI allow rapid screening of large chemical libraries in very small quantities and subsequent structural activity relationship (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 pre-clinical studies for evaluation of the efficacy of different HDACi in small animal models. For example, the HDAC inhibitor SAHA was radiolabeled with $^{18}$F for PET/CT imaging of ovarian
carcinomas treated with SAHA (47), while $^{18}$F-FLT PET has been used to monitor the effect of LAQ824 and PXD101 on cell proliferation in colon carcinomas [58, 59]. Using the same cohort of mice for BLI imaging, we also monitored the inhibition of glucose metabolism by 1A12 using $^{18}$F-FDG (Figure 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 HDAC inhibitors 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.
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Figure Legends:

Figure 1: Monitoring of the efficacies of HDAC inhibitors (HDACi) using the Hsp90(α/β)/p23 split RL reporter system. (A) Schematics of the split RL reporter system. The two interacting proteins p23 and Hsp90(α/β) were fused to the ‘NRL’ (aa 1-229 with a M185V mutation to increase light output) and ‘CRL’ (aa 230-311) portion of the RL through a peptide linker, respectively (top). In the presence of ATP, interactions between p23/Hsp90 brought ‘NRL’ and ‘CRL’ in close proximity and led to complementation of RL enzyme activities, and photon production in the presence of the substrate coelentarazine or Enduren™. RL activities were measured by optical bioluminescence imaging (BLI) of intact cells in cell culture and in living mice using a cooled CCD camera using Enduren™ and coelentarazine, respectively. Inhibition of HDAC activities by HDACi (red cross) leads to hyperacetylation of Hsp90 by histone acetyl-transferase (HAT). This modification of Hsp90(α/β) leads to disruption of its interaction with p23 and thus reduced complemented RL activities and photon production. (B-D): Disruption of Hsp90(α/β)/p23 interactions by pan- HDAC inhibitors in cell culture. 293T cells stably expressing Hsp90(α/β)/p23 split RL reporters were treated with different concentrations of pan-HDAC inhibitor TSA (B), VPA (C) and APHA compound 8 (D) or their respective carrier controls for 24 hours. Complemented RL activities were determined by BLI of intact cells 90 mins upon addition of the RL substrate Enduren™ using a cooled CCD camera (left). Viable cell number in each well was determined by SRB assay. Bioluminescence signals at each inhibitor concentration were normalized to cells number and to carrier control (right).

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 HDAC-1 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 Hsp90 inhibitor in combination with HDAC6 inhibitor on inhibition of Hsp90(α/β)/p23 interactions, stable cells were treated with Tubacin or Niltubacin along with 250 nM PU-H71 for 24 hours, prior to determination of complemented RL activities by BLI as in Figures 1B-D. Tubacin in combination with PU-H71 led to greater inhibition of Hsp90α/p23 interactions compared to 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.

**Figure 3: Specificity of using dual-reporter system for monitoring the efficacy of HDAC inhibitors.** To determine the specificity of using the dual-reporter system for non-invasive monitoring the efficacies of HDAC inhibitors, 293T cells were treated with the indicated HDAC inhibitors for 24 hours, prior to Western blotting of the expression of p21\textsuperscript{waf1}, Hsp70, acetylated Histone H3 and acetylated α-tubulin. The blots were stripped and reprobed with α-tubulin antibody to control for protein loading.

**Figure 4. Chemical syntheses and *in vitro* screening of novel HDAC inhibitors.** (A) Schematic of synthesis of novel HDAC inhibitors. To explore the effect of cap space on inhibition of HDAC, cinnamic hydroxamates were capped with different aldehyde (Supplemental Figure S3A) and ketone groups. (B) Evaluation of the top 384 lead compounds from (A) for their efficacy in disrupting Hsp90(α/β)/p23 interactions in intact cells. 293T cells stably expressing the dual reporter system were treated with the lead compounds at 4.5 μM (rows A-F, columns 1-12) or carrier control (row H, columns 1-12). Their effects on Hsp90(α/β)/p23 interactions and cell proliferation were monitored by sequential RL and FL imaging upon, respectively. Cells treated 5 μM PU-H71 (row G1-7) or 30 μM WT161 (row G8-12) served as positive controls. Results from RL imaging of 293T cells expressing Hsp90α/p23 reporters were shown here. The
lead compounds were circled in white.

**Figure 5:** Characterization of the lead HDAC inhibitors. (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 hrs. prior to RL imaging. WT161 was used a positive control. (C) Net effect of 1A12 (squares), 2C2 (triangles) and 2H9 (diamonds) on Hsp90(α/β)/p23 interactions in intact cells. The effect of these compounds on cell proliferation was determined by FL imaging as in Figure N1c. Normalization of RL to FL signals shows that 1A12 led to similar levels of inhibition of Hsp90(α/β)/p23 interactions compared to that of WT161 (circles), and were more effective than 2C2 and 2H9. Furthermore, all the compounds were more selective for inhibition of Hsp90α/p23 interactions, compared to that of Hsp90β/p23 interactions. (D) Up-regulation of biomarkers of HDAC inhibition by lead compounds. To validate the mechanisms of the lead compounds at the doses that inhibits Hsp90(α/β)/p23 interactions, 293T cells were treated with 2H9, 1A12 and 2C2 or carrier control at the indicated concentrations for 24 hours, prior to western blotting for the expression of p21\textsuperscript{waf1}, act-H3 and Hsp70. WT161 (30 μM) and PU-H71 (5 μM) served as positive controls while α-tubulin was used a loading control. 1A12 was the most effective compound that led to dose-dependent increase in the level of the HDAC biomarkers (p21\textsuperscript{waf1}, acetylated H3) and Hsp90 biomarkers (Hsp70), followed by 2C2 and 2H9.

**Figure 6:** 1A12 disrupts Hsp90(α/β)/p23 interactions in 293T tumor xenografts in mice. (Top) To determine the effect of 1A12 on Hsp90(α/β)/p23 interactions and cell proliferation in living mice, baseline RL and FL signals in 293T xenografts expressing Hsp90α/p23 (left tumors) and Hsp90β/p23 dual reporter system (right tumors) were determined
by optical bioluminescence imaging. Mice were then randomized into two groups and i.p. injected with 80 mg/kg of 1A12 (N = 8) or carrier control (N = 10). Mice were re-imaged at 15 hours after the initial dose and re-imaged at 40 hours after two more doses of 1A12. (Bottom) RL signals were normalized to that of FL signals at each time point and to time 0 hr to determine the net effect of 1A12 on Hsp90(α/β)/p23 interactions. 1A12 led to decrease in Hsp90(α/β)/p23 interactions. *, p < 0.0014 vs. carrier control treated mice.

**Figure 7: 1A12 inhibits glucose metabolism in Hsp90(α/β)/p23 tumor xenografts in mice.**

(A) The effect of 1A12 on glucose metabolism in tumor xenografts was determined by small animal PET/CT imaging in conjunction with 18F-FDG. Baseline 18F-FDG uptake was established prior to treatment with 3 doses of 80 mg/kg of 1A12 or carrier control over period of 40 hours. Mice were re-imaged on Day 2. The dotted lines indicate the location of the Hsp90α/p23 (left) and Hsp90β/23 (right) tumor xenografts. (B) The % increase in 18F-FDG uptake relative to Day 0 was expressed as mean and max %ID/g of injected dose. Compared to carrier control, the mean increase in 18F-FDG uptake was significantly reduced in mice treated with 1A12 (p = 0.01).
**RL Imaging:**
Hsp90(α/β)/p23 Interactions

**FL Imaging:**
Cell Proliferation

<table>
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<th>Time post initial 1A12 treatment (Hours)</th>
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Hsp90α/p23  | Carrier Control  | Hsp90β/p23  | 1A12 (80 mg/kg)

**Graphs:**

- **Net Hsp90α/p23 Interactions**
- **Net Hsp90β/p23 Interactions**
Syntheses and Discovery of a Novel Class of Cinnamic Hydroxamates as Histone Deacetylase (HDAC) Inhibitors by Multimodality Molecular Imaging in Living Subjects

Carmel T Chan, Jun Qi, William Smith, et al.

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