OSU-HDAC42, a Histone Deacetylase Inhibitor, Blocks Prostate Tumor Progression in the Transgenic Adenocarcinoma of the Mouse Prostate Model

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Abstract
Histone deacetylase (HDAC) inhibitors suppress tumor cell growth via a broad spectrum of mechanisms, which should prove advantageous in the context of cancer prevention. Here, we examined the effect of dietary administration of OSU-HDAC42, a novel HDAC inhibitor, on prostate tumor progression in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Based on a series of pilot studies, an AIN-76A diet was formulated containing 208 ppm OSU-HDAC42, which was estimated to deliver ~25 mg/kg of drug per day to each mouse and found to cause a suppression of PC-3 xenograft tumor growth equivalent to that achieved by gavage administration of a similar dose. At 6 weeks of age, TRAMP mice received this drug-containing or control diet for 4 or 18 weeks and were evaluated for prostatic intraepithelial neoplasia (PIN) and carcinoma development, respectively. OSU-HDAC42 not only decreased the severity of PIN and completely prevented its progression to poorly differentiated carcinoma (74% incidence in controls versus none in drug-treated mice), but also shifted tumorigenesis to a more differentiated phenotype, suppressing absolute and relative urogenital tract weights by 86% and 85%, respectively, at 24 weeks of age. This tumor suppression was associated with the modulation of intraprostatic biomarkers, including those indicative of HDAC inhibition, increased apoptosis and differentiation, and decreased proliferation. With the exception of completely reversible hematomic alterations and testicular degeneration, no significant changes in body weight or other indicators of general health were observed in drug-treated mice. These results suggest that OSU-HDAC42 has value in prostate cancer prevention.

Introduction
Prostate cancer is the most commonly diagnosed noncutaneous cancer and the second leading cause of cancer death in men (1). Prostatic tumors often have a long initial latency period before becoming pathologically advanced, at which point they are invariably fatal (2, 3). Unfortunately, a consistently deregulated group of genes critical for malignant progression has not been identified for this disease (4). These attributes suggest a need to prevent prostate cancer, such as by chemopreventive agents capable of simultaneously modulating multiple cellular processes involved in prostate carcinogenesis.

Histone deacetylase (HDAC) inhibitors are appropriate candidates in this regard because of their ability to selectively induce apoptosis (5, 6), cell cycle arrest (7–9), and differentiation (10, 11) in cancer cells. These compounds have shown pleiotropic anticancer activities in many recent preclinical and clinical investigations of human cancers, including prostate cancer, through both histone acetylation–dependent and histone acetylation–independent mechanisms (10, 12–14). First, and representing the conventional rationale for their development, HDAC inhibitors induce the reexpression of growth arrest genes silenced in cancer through chromatin remodeling (15). Second, HDAC inhibitors modulate a growing list of nonhistone HDAC substrates including transcription factors and mediators of cell signaling in cancer cells, the immune system, and tumor vasculature (12, 16, 17). The paucity of knowledge of HDAC biology, however, has precluded a complete understanding of the anticancer mechanisms of these agents, and the toxicologic sequelae of chronic therapy are largely unknown (18).

We recently reported our development of a novel phenylbutyrate-based HDAC inhibitor, OSU-HDAC42, and showed its potent and pleiotropic anticancer activities in models of human prostate and hepatic cancer in vitro and in vivo (19–24). Specifically, OSU-HDAC42 induces hallmark indicators of HDAC inhibition, including histone H3 acetylation, α-tubulin acetylation, and p21 up-regulation, and modulates regulators of cell survival, such as Akt, Bcl-xl, Bax, Ku70, survivin, and members of the inhibitor of apoptosis protein family (19–21, 24, 25). In the present study, to evaluate these tumor suppressive effects in a chemopreventive context, we extended the use of OSU-HDAC42 to the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, which mimics spontaneous tumor progression in man (25). Prostate lesion development in TRAMP mice is driven by the SV40 large T antigen (TAg) in the prostate epithelium (26). TAg disrupts cellular p53 and retinoblastoma functions and is expressed upon activation of an androgen-response element in the probasin promoter (26). The ensuing tumorigenesis occurs in an age-dependent, multistage process, including hyperplasia, prostatic intraepithelial neoplasia (PIN), and adenocarcinoma with distant metastasis (27).

Here, we use an interdisciplinary approach entailing medicinal chemistry, molecular pharmacology, and veterinary pathology to assess the chemopreventive potential of OSU-HDAC42 concurrent with an investigation of whole-body ramifications of chronic therapy by using TRAMP mice as a model. Based on pilot studies...
demonstrating that dietary administration of OSU-HDAC42 suppressed PC-3 xenograft tumor growth, the drug was given via diet to TRAMP mice to evaluate its inhibitory effects on PIN and prostate carcinoma. Collectively, our results show that OSU-HDAC42 achieves a remarkable suppression of prostate tumorigenesis in the absence of limiting toxicity.

Materials and Methods

Reagents. The HDAC inhibitors vorinostat (also known as suberoylanilide hydroxamic acid, Zolinza, Merck & Co., Inc.) and OSU-HDAC42 (also known as (S)-(+) - N-hydroxy-4-(3-methyl-2-phenylbutyramino)-benzamido) were synthesized in our laboratory with purities exceeding 99% as shown by nuclear magnetic resonance spectroscopy (300 MHz). OSU-HDAC42 (NSC D736012) is a novel hydroxamate-tethered phenylbutyrate derivative that had been evaluated preclinically by the Rapid Access to Intervention Development program at the National Cancer Institute (NCI). Vorinostat is the first HDAC inhibitor to earn U.S. Food and Drug Administration (FDA) approval and is marketed for the treatment of cutaneous T-cell lymphoma (10). AIN-76A rodent diet with and without 208 ppm OSU-HDAC42 was obtained from Research Diets, Inc. Mouse monoclonal antibodies against various target proteins were obtained from the following sources: p21 and TAg (Pub 101; Santa Cruz Biotechnology, Inc.), α-tubulin and acetylated (Ac) α-tubulin (Sigma-Alrich), synaptophysin (Dako North America, Inc.), and β-actin (ICN Biomedicals). Rabbit polyclonal antibodies against various target proteins were obtained from the following sources acetylated histone H3 (Ac-H3; Upstate Biotechnology, Inc.) and Bax, Ki67, cleaved caspase-3, and E-cadherin (Cell Signaling Technology, Inc.).

Animals. Male NCr athymic nude mice (5–7 wk of age) were obtained from the NCI and injected s.c. with PC-3 cells, as described (21). Treatment was initiated 7 d after injection, with the gavage dose of drug adjusted twice weekly to mirror the dose of drug consumed in the diet. TRAMP mice (C57BL/6TRAMPxFVB) were generated and housed as previously reported (28). The reversibility of observed OSU-HDAC42-associated toxicity was determined in age-matched nontransgenic (wild-type) littermates of TRAMP mice, which included comparison of weights of the adrenal glands, brain, epididymides, epididymal fat pads, heart, kidneys, liver, pituitary gland, spleen, testes, and thymus. The procedures performed were in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Histopathology. The entire carcass of each animal in all studies was evaluated for gross lesions and preserved in 10% formalin at necropsy. An extended set of tissues from representative animals (n = 3, 5, and 10) for the pharmacodynamic, xenograft, and TRAMP studies, respectively was evaluated microscopically in accordance with Society of Toxicologic Pathology–proposed guidelines for repeat-dosing toxicity studies (29), with the exception of spinal cord and female reproductive organs. Dorsal (DP), lateral (LP), ventral (VP), and anterior (AP) prostate lobes, iliac lymph nodes, liver (left lobe), and lungs from each TRAMP mouse were collected, fixed, processed, and evaluated as described (28). Testes were fixed for 24 h in Bouin’s fixative and then transferred to 70% ethanol. The observer scoring the prostate slides (A.S.), via a TRAMP-specific grading scheme (30), was blinded to the treatment status.

Immunodetection of biomarkers. TRAMP prostate tissue homogenates were prepared and immunoblotted against Ac-H3, p21, Ac-α-tubulin, α-tubulin, E-cadherin, synaptophysin, Bax, TAg, and β-actin as described (28). Immunohistochemical detection of Ac-H3, Ki67, cleaved caspase-3, E-cadherin, and synaptophysin was performed on 4-μM-thick, paraffin-embedded PC-3 xenograft tumor, TRAMP prostate, testis, spleen, and/or bone marrow tissue sections in accordance with the manufacturers’ recommendations.

Statistical analysis. Satisfying the requisites of independence and normal distribution, a Student’s t test was used to determine if certain responses (immunohotblotting and immunohistochemistry results, xenograft tumor volumes, body and tissue weights, and microscopic lesion scores and distributions) were influenced by drug treatment. The incidences of prostate lesions and metastasis were compared with a χ² contingency analysis. Differences between groups were considered significant at P < 0.05.

Results

Oral administration of OSU-HDAC42 modulates targets in TRAMP prostate with higher potency relative to vorinostat. A series of pilot studies was carried out to identify an optimal dose and administration route of OSU-HDAC42 in preparation for a long-term prevention study in TRAMP mice. Considering the effect of dietary restriction on suppressed tumorigenesis in TRAMP mice (30, 31), a dose-ranging study was performed to identify a dosing schedule of OSU-HDAC42 that would not compromise body weight. Our data indicate that the doses of 25 mg/kg everyday or 50 mg/kg every other day by gavage had no significant effect on body weight and exhibited no limiting toxicity in TRAMP mice. However, progressive weight losses were observed at higher doses, including 40 mg/kg everyday and 65 mg/kg every other day (data not shown).

To assess the HDAC inhibitory potency of these tolerated doses of OSU-HDAC42 in vivo, their pharmacodynamic effects vis-à-vis those of vorinostat on intraprostatic levels of histone H3 acetylation and p21 expression in the DP of TRAMP mice were evaluated by Western blotting. Treatment with OSU-HDAC42 at both doses for 14 days caused greater increases in histone H3 acetylation than vorinostat at 50 mg/kg everyday (P < 0.05). A similar, but not statistically significant, trend occurred in p21 expression levels between OSU-HDAC42 and vorinostat (Fig. 1A). Together, these data indicate a higher HDAC inhibitory potency for OSU-HDAC42 than for vorinostat at these dosing schedules.

Dietary and gavage administrations of OSU-HDAC42 achieve equivalent suppression of PC-3 xenograft tumor growth. To avoid repeated gavage-associated stressing of mice, feeding of drug in the diet represented a preferred route for long-term drug administration. To examine the feasibility of dietary delivery, the effect of OSU-HDAC42 given via gavage versus diet on PC-3 tumor growth in nude mice was assessed. Accordingly, a diet was formulated to contain 208 ppm OSU-HDAC42, a concentration estimated by food consumption measurements to achieve an average dose of 25 mg/kg/d, which was previously reported to suppress PC-3 tumor growth when given by gavage (21). PC-3 subcutaneous xenograft-bearing nude mice were treated with this diet or by daily gavage (with the gavage dose of OSU-HDAC42 adjusted twice weekly to mirror that consumed in the diet) for 6 weeks and analyzed with respect to tumor volume and intratumoral target modulation.

The tumor growth curves for animals treated with drug in the diet and by gavage were nearly overlapping (Fig. 1B). Diet-administered and gavage-administered drug equivalently suppressed tumor volumes by 65.6% and 65.3%, respectively, compared with control animals (respective final volumes of 190 ± 17 mm³ and 183 ± 29 mm³ versus 424 ± 51 mm³ in controls, mean ± SE, P < 0.002). The combined weights of the testes/epididymides were also comparably decreased by 64.5% and 61.8% in gavage-treated and diet-treated mice, respectively (101 ± 8.4 mg and 108 ± 13 mg versus 284 ± 26 mg in controls, mean ± SD, P < 0.0001). Equivalent effects on blood cell counts and serum chemistry were also observed (data not shown; effects discussed in results of TRAMP studies below). Dietary drug treatment did not significantly affect terminal body weight (30.3 ± 3.8 g in control mice versus...
30.4 ± 2.5 g in treated mice, mean ± SD). Over the course of the study, mice consumed 3.9 ± 0.2 g of diet per day, representing a 28 ± 3.3 mg/kg/d dose of OSU-HDAC42 (means ± SD).

Suppression of PC-3 xenograft tumor growth is associated with target modulation by diet-administered OSU-HDAC42.

We previously reported that OSU-HDAC42 modulated a series of targets governing multiple aspects of prostate cancer cell survival in vitro and in vivo (21). To link the tumor suppressive activity of diet-administered OSU-HDAC42 with its effects on relevant biomarkers, immunohistochemical analyses were performed and indicate that the drug treatment significantly decreased the proliferation marker Ki67 concurrent with increased staining for Ac-H3 and the apoptosis marker cleaved caspase-3 (Fig. 1C and D).

TRAMP mice develop prostatic hyperplasia and PIN at 6 weeks of age. Having confirmed that the dietary administration of OSU-HDAC42 at 208 ppm was effective and safe in PC-3 tumor-bearing mice, we assessed its chemopreventive activity in the TRAMP model. Because TRAMP mice display an early stage of androgen-driven tumorigenesis by 6 weeks of age (26, 32), treatment was started at this age in our prevention study. The extent of lesions in prostate tissues of 6-week-old TRAMP mice was compared with that of age-matched wild-type mice with respect to urogenital tract (UGT) and prostate lobe weights, and microscopic lesion score and intralobe distribution. The weights of TRAMP UGT and prostate tissue were higher than that of wild-type mice, and this increase was attributable to epithelial hyperplasia and PIN, most notably in the DPs and LPs (Fig. 2A; Supplementary Fig. S1).

OSU-HDAC42 decreases the severity of PIN in TRAMP mice at 10 weeks of age. PIN represents an opportune intervention point in man at which an effective therapeutic strategy could theoretically prevent or slow the progression to carcinoma (33–35). To assess the ability of OSU-HDAC42 to delay PIN development, 6-week-old TRAMP mice were treated with either control AIN-76A rodent diet or the diet containing 208 ppm OSU-HDAC42 through 10 weeks of age when control animals are expected to have developed advanced PIN and a low incidence of carcinoma (25). An approximate delivery of 19 mg OSU-HDAC42/kg/d to TRAMP mice over the course of the study was estimated by food consumption measurements.

PIN remained the most severe lesion in TRAMP mice after 4 weeks of treatment in both groups, with the exception of one poorly differentiated tumor in a control diet–fed mouse (Table 1). To ascertain subtle differences in lesion development between treatment groups, the weights of UGTs and prostate lobes, and microscopic lesion scores and intralobe distributions were
compared. Figure 2B and C show that OSU-HDAC42 suppressed
the overall severity of PIN lesions as manifested in significant
reductions in the weights of the UGTs and DPs and significant
decreases in the microscopic lesion scores in the LPs and VPs.
The distribution of lesions within each prostate lobe was also
affected, with a trend, as evident in the LPs and VPs and
correlating with the decreased lesion score in these lobes, toward
more normal epithelium and hyperplasia, and less PIN in drug-
treated TRAMP mice (Fig. 2D). Seminal vesicle weights were also
decreased by drug treatment [169 ± 32 mg in treated mice
(n = 15) versus 193 ± 31 mg in control mice (n = 15), mean ±
SD, P < 0.05].

OSU-HDAC42 prevents the progression of PIN to poorly
differentiated carcinoma and shifts tumorigenesis to a more
differentiated phenotype in TRAMP mice at 24 weeks of age.
We hypothesized that the suppressive effect of OSU-HDAC42 on
the development of early proliferative lesions in TRAMP mice, as
described above, would retard or prevent progression to long-term
carcinoma. Accordingly, a larger numbers of animals (n = 23, to
account for the expected increase in overall variation in lesion
development in controls with age) were treated from 6 to 24 weeks
of age when the majority of TRAMP mice will have developed
advanced, metastatic tumors (25).

As summarized in Table 1, OSU-HDAC42 completely prevented
the occurrence of macroscopic prostate tumors (78% of controls
versus none in the drug-treated group) and of poorly differentiated
carcinoma (74% of controls versus none in the drug-treated group)
after 18 weeks of treatment. Only 1 of 23 drug-treated TRAMP
mice had evidence of carcinoma microscopically (moderate dif-
ferration). This profound inhibitory effect was reflected in the
86% and 85% reductions in absolute and relative UGT weights,
respectively, in OSU-HDAC42 diet–fed mice relative to the controls.
Specifically, the mean absolute and relative values of control versus
drug-treated UGT weights were 3.1 g versus 0.4 g and 10% versus
1.5%, respectively (Fig. 3A and B; Table 1). The similarity in the
reductions in absolute and relative UGT weights shows that the
antitumor effects occurred independent of an effect on body
weight. Interestingly, the relative UGT weight of drug-treated
TRAMP mice at 24 weeks of age was the same as that of control
TRAMP mice at 10 weeks of age (1.5% of body weight).

Analysis of individual prostate lobes indicates that OSU-HDAC42
caused significant reductions in the weights of the DPs and

Figure 2. OSU-HDAC42 suppressed the development of prostate lesions in 10-wk-old TRAMP mice. A, photomicrographs of representative DP tissue from
TRAMP and wild-type mice at the beginning of treatment (6 wk of age; H&E stain, 200× magnification). Note the histologic spectrum of normal epithelium, simple
hyperplasia, and PIN in the TRAMP prostate at 6 wk. B, UGT and individual prostate lobe weights of TRAMP mice fed an AIN-76A rodent diet with or without
208 ppm OSU-HDAC42 from 6 to 10 wk of age, compared with wild-type littermates fed a control diet. C, scoring of lesion severity in individual prostate lobes of control
and OSU-HDAC42–treated TRAMP mice. D, distribution of lesions within the prostate lobes of control and OSU-HDAC42–treated TRAMP mice. Columns, mean;
bars, SD (n = 15 TRAMP and 5 wild-type mice). *, P < 0.05; **, P < 0.01. HDAC42, OSU-HDAC42; N, normal; HP, hyperplasia; AD, adenoma; WD, well-differentiated
carcinoma; MD, moderately differentiated carcinoma; PD, poorly differentiated carcinoma; PHY, phyllodes-like tumor.
Control TRAMP mice developed palpable prostate tumors at 14.7 weeks of age, whereas drug treatment [274 mg/kg body weight (BW)] significantly delayed the appearance of tumors at 24 weeks of age. In general, seminal vesicle weights were also decreased in drug-treated mice compared with controls, indicating a decrease in sperm production. Hyperplastic areas, indicative of neoplastic growth, were present in all prostate lobes compared with controls, suggesting a shift to a more differentiated phenotype in response to OSU-HDAC42. These areas were characterized by increased size, number, and complexity of glandular structures. The marked disparity in prostate morphology at 24 weeks of age between the two treatment groups, i.e., poorly differentiated tumors in controls versus PIN and adenomas in drug-treated mice, suggested a shift to a more differentiated phenotype in response to OSU-HDAC42. Whereas this disparity precluded a morphology-matched comparison of biomarkers between treatment groups, an effort was made to evaluate the expression of biomarkers of differentiation in representative tissue samples by using immunohistochemistry and immunoblotting analyses. Distinct differences in the expression levels of E-cadherin and synaptophysin were noted between the treatment groups, both of which were reported to be dysregulated with lesion progression in TRAMP mice (25, 38). These differences were observed in the PIN lesions of TRAMP mice after administration of OSU-HDAC42. The expression of E-cadherin, which is a marker of epithelial phenotype, was significantly increased in OSU-HDAC42-treated mice compared with controls (Fig. 4A). The expression of synaptophysin, a marker of neuroendocrine phenotype, was significantly decreased in OSU-HDAC42-treated mice compared with controls (Fig. 4B). In addition, the expression of Ki67, a marker of cell proliferation, was significantly decreased in OSU-HDAC42-treated mice compared with controls (Fig. 4C). The expression of cleaved caspase-3, a marker of apoptosis, was significantly increased in OSU-HDAC42-treated mice compared with controls (Fig. 4D).

Table 1. Relative UGT weight and incidence of prostate lesions in TRAMP mice treated from 6 to 10 or 24 wk of age

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Relative UGT weight*</th>
<th>Macrophoscopic tumors</th>
<th>Most severe lesion in any prostate lobe</th>
<th>Metastases</th>
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<tr>
<td></td>
<td></td>
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<td>PIN</td>
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<td>Weeks</td>
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<tr>
<td>10</td>
<td>15</td>
<td>1.5 ± 0.0</td>
<td>1 (6.7%)</td>
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<tr>
<td></td>
<td>15</td>
<td>1.2 ± 0.0a</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>23</td>
<td>1.0 ± 0.7</td>
<td>18 (78.3%)</td>
<td>3 (13%)</td>
<td>1 (4.3%)</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>1.5 ± 0.0a</td>
<td>0b</td>
<td>12 (52.2%)c</td>
<td>10 (43.5%)d</td>
</tr>
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</table>

NOTE: Incidence refers to the number of animals affected with percentage of total indicated in parentheses. Difference from control group is significant at *P < 0.001 by Student’s t test and †P < 0.001, and ‡P < 0.05 by χ² analysis. Abbreviations: AD, adenoma; WD, well-differentiated carcinoma; MD, moderately differentiated carcinoma; PD, poorly differentiated carcinoma; LN, lymph node; HDAC42, OSU-HDAC42.

*Values represent percentages calculated as UGT weight / body weight × 100, mean ± SE; values at 6 wk of age (initiation of treatment) = 0.9 ± 0.0. †Lung and/or liver.

microscopic lesion scores of the DPs, LPs, and VPs (Fig. 3B and C). As manifested in the lesion scores, the breakdown of lesion distribution by lobe shows that PIN remained the predominant lesion in the majority of OSU-HDAC42–treated mice at 24 weeks of age (Fig. 3D). In addition to the absence of poorly differentiated tumors, drug-treated mice had significant increases in normal, hyperplastic, and/or PIN in all prostate lobes compared with control diet–fed mice. Seminal vesicle weights were also decreased by drug treatment [274 ± 62 mg in treated mice (n = 23)] versus 377 ± 147 mg in control mice (n = 22); mean ± SD, P = 0.01]. Control TRAMP mice developed palpable prostate tumors at 19 ± 2.7 weeks of age; the tumors, including urinary bladder, weighed 3.4 ± 2.1 g at the time of sacrifice. Interestingly, the incidence of adenomas at 24 weeks of age was greater in drug-treated TRAMP mice (43.5% versus 4.3% of controls; Table 1), and all adenomas occurred in the DPs (Fig. 3D). These tumors displayed characteristics consistent with adenoma according to terminology proposed by the Mouse Models of Human Cancer Consortium Prostate Pathology Committee (36). They consisted of focally distinct, intraluminal projections of well-differentiated epithelium arranged in papillary projections on hypocellular and nonedematous connective tissue scaffolds (Fig. 4C, bottom left). A supportive fibrovascular stalk and compression atrophy of adjacent epithelium were present in some sections. Nuclear and cellular atypia, mitotic figures, and destructive invasion were lacking. Arising within a background of PIN, the adenomas generally occupied a small percentage of total lobe area (8.3 ± 2.5%, mean ± SE).

To shed light onto the latency of the adenomas after agent withdrawal, two TRAMP mice were returned to control diet at 24 weeks of age. These mice were sacrificed at 42 weeks of age with palpable tumors arising in the seminal vesicles that were determined microscopically to be epithelial-stromal tumors (37). The prostate lobes were variably expanded by phyllodes-like tumors (APs, DPs), PIN (VPs), and well-differentiated carcinoma (WDs) with no evidence of metastasis (Supplementary Fig. S2).

Tumor suppression in TRAMP mice by OSU-HDAC42 is associated with target modulation and biomarkers of differentiation. Relevant HDAC inhibition-associated and cell survival biomarkers were assessed in the PIN lesions of TRAMP mice after treatment from 6 to 10 weeks of age. Drug treatment significantly altered the proliferation and apoptotic indices in PIN, as shown by immunohistochemical staining of Ki67 and cleaved caspase-3 (Fig. 4A). OSU-HDAC42 induced 2.6-fold and 2-fold increases (P < 0.01) in the acetylation of α-tubulin and the expression of Bax compared with controls, respectively (Fig. 4B). An increase in histone H3 acetylation was also noted, but was not statistically significant due to greater variation in this biomarker.

The marked disparity in prostate morphology at 24 weeks of age between the two treatment groups, i.e., poorly differentiated tumors in controls versus PIN and adenomas in drug-treated mice, suggested a shift to a more differentiated phenotype in response to OSU-HDAC42. Whereas this disparity precluded a morphology-matched comparison of biomarkers between treatment groups, an effort was made to evaluate the expression of biomarkers of differentiation in representative tissue samples by using immunohistochemistry and immunoblotting analyses. Distinct differences in the expression levels of E-cadherin and synaptophysin were noted between the treatment groups, both of which were reported to be dysregulated with lesion progression in TRAMP mice (25, 38). In 24-week-old TRAMP mice, OSU-HDAC42 maintained E-cadherin expression, which was lost in controls, and prevented the expression of synaptophysin (an indicator of the neuroendocrine phenotype), which was increased in controls (Fig. 4C and D). The increased Ac-H3 levels in adenomas confirmed the HDAC inhibitory effect of OSU-HDAC42 in the prostate (Fig. 4C).

OSU-HDAC42 causes reversible hematologic alterations and testicular degeneration. Toxicologic effects of OSU-HDAC42 in TRAMP mice were assessed by hematologic, histopathologic, and body and organ weight evaluations. There was no significant difference in the body weight after 18 weeks of treatment [31 ± 3.6 g for treated mice (n = 23) versus 29 ± 2.7 g for control mice (n = 23), mean ± SD, P = 0.6]. The weights of epididymal fat pads, measured as an assessment of body fat and additional indicator of general health, were also not significantly altered [549 ± 349 mg in treated mice (n = 23) versus 625 ± 282 mg in control mice (n = 18), mean ± SD, P = 0.5]. After 4 weeks of treatment with OSU-HDAC42 (i.e., 10 weeks of age), a significant leukopenia, characterized by a mature neutropenia and lymphopenia, was present, and red cell numbers were decreased (Supplementary Table S1). All blood cell counts returned to control levels upon withdrawal of drug in wild-type littermates of TRAMP mice. Femoral and sternebral bone marrow of 4 week–treated animals was of comparable cellularity.
and proliferative status to controls with all cell types and stages of maturation present (Supplementary Fig. S3). None of the hematologic abnormalities observed after 4 weeks of treatment was noted after 18 weeks of treatment, i.e., 24 weeks of age (Supplementary Table S1). Extramedullary hematopoiesis (EMH) was increased in the spleens of mice treated for both 4 and 18 weeks, and reversible thymic atrophy, shown by histology and organ weight data, occurred in association with OSU-HDAC42 treatment (Supplementary Fig. S3 and Supplementary Table S2).

Significant effects on serum chemistry after 4 weeks of treatment included increased aspartate aminotransferase, total bilirubin, and albumin and decreased alkaline phosphatase (ALP) and cholesterol, all of which, like the blood cell count alterations, returned to control levels upon discontinuation of the drug. At 18 weeks of treatment, significant decreases remained in ALP and cholesterol, as well as decreased potassium and increased protein that seemed to be due to elevated albumin (Supplementary Table S1).

Other than effects on the prostate, spleen (EMH), and thymus (atrophy), the only lesion detected by gross or histopathologic examination was severe testicular degeneration in drug-treated mice. A marked bilateral reduction in the size of the testes was noted after 2 weeks of repeated dosing of OSU-HDAC42, but not with vorinostat (Fig. 5A). Weights of the testes and epididymides decreased progressively in TRAMP mice after 4 and 18 weeks of treatment and eventually returned to control levels upon withdrawal of the drug after each treatment period (Fig. 5C). Mice were able to achieve litters within 6 to 8 weeks of drug withdrawal after both 4 and 18 weeks of treatment, and the sizes of these litters (9 and 11 pups for the first litters of two recovered mice after 4 weeks of treatment and 8 and 13 pups for the first litters of two recovered mice after 18 weeks of treatment) were estimated to be normal for their strain background.

Histologically, the seminiferous tubules of drug-treated mice were devoid of spermatozoa and lined predominantly by Sertoli cells, spermatogonia, and variable numbers of primary spermatocytes up to the leptotene-zygotene stage. Immunostaining of Ki67 shows that actively dividing spermatogonia remained even after 18 weeks of drug treatment (Fig. 5B). No morphologic abnormalities of the Sertoli or Leydig cells were noted.

Tumor suppression by OSU-HDAC42 in TRAMP mice is not associated with disruption of large TAg expression. Given the consistent and marked drug effect on the testes, an effort was made to determine whether the dramatic suppression of tumorigenesis in TRAMP mice was associated with disruption of TAg expression or androgen production. Immunoblotting of prostate tissue homogenates shows that TAg was expressed at similar levels decreased progressively in TRAMP mice after 4 and 18 weeks of treatment and eventually returned to control levels upon withdrawal of the drug after each treatment period (Fig. 5C). Mice were able to achieve litters within 6 to 8 weeks of drug withdrawal after both 4 and 18 weeks of treatment, and the sizes of these litters (9 and 11 pups for the first litters of two recovered mice after 4 weeks of treatment and 8 and 13 pups for the first litters of two recovered mice after 18 weeks of treatment) were estimated to be normal for their strain background.
Figure 4. Histology and immunochemical analysis of biomarkers in the prostates of TRAMP mice fed an AIN-76A rodent diet with or without 208 ppm OSU-HDAC42 from 6 to 10 (A and B) or 24 (C and D) wk of age. A, immunohistochemical evaluation of Ki67 and cleaved caspase-3 in prostates of TRAMP mice treated via experimental diet as described above. Decreased Ki67 and increased cleaved caspase-3 immunopositivities (400× magnification) were associated with a lesser degree of epithelial proliferation in the LPs of drug-treated TRAMP mice (score 8) compared with controls (score 9; H&E stain, 200× magnification). The percentages of positive-staining cells were 20.7 ± 3.1 versus 13.9 ± 3.1 for Ki67 (P < 0.01) and 1.4 ± 1.0 versus 2.0 ± 1.0 for cleaved caspase-3 (P < 0.05) in control versus drug-treated prostate, respectively (means ± SD from 10 randomly chosen 400× magnification fields). B, representative immunoblots of prostate tissue lysates show increases in the acetylation of α-tubulin and histone H3, and Bax expression in OSU-HDAC42–treated TRAMP mice. The values in fold, quantified by densitometry, denote the intensity of protein bands relative to that of α-tubulin (Ac-α-tubulin) or β-actin (Ac-H3, Bax) and standardized to the control diet group. Means ± SD (n = 3). The fold increases in Ac-α-tubulin and Bax were significant at P < 0.01. C, immunohistochemical evaluation of differentiation markers (E-cadherin and synaptophysin) and Ac-H3 in prostates of TRAMP mice treated via experimental diet as described above. H&E–stained sections show a poorly differentiated carcinoma (score 18, 200× magnification) and an adenoma (score 13, 100× magnification) in the DPs of control and drug-treated TRAMP, respectively. Immunohistochemistry (400× magnification) shows that this tumor suppression was associated with retention of E-cadherin and prevention of synaptophysin expression and increased acetylation of histone H3. D, immunoblotting of prostate tissue lysates confirms the effects of OSU-HDAC42 on E-cadherin and synaptophysin expression shown immunohistochemically in C.
Figure 5. Characterization of OSU-HDAC42-associated testicular degeneration. A, photograph showing the representative gross appearances of testes/epididymides from TRAMP mice treated as indicated for 14 d. Note the reduction in size of OSU-HDAC42–treated, but not vorinostat-treated, testes. B, histologic evaluation of the seminiferous tubules of control and OSU-HDAC42–treated TRAMP mice. The tubules of drug-treated mice are lined by Sertoli cells, spermatogonia (Ki67 immunopositive cells), and variable numbers of spermatocytes up to the leptotene-zygotene stage (Bouin’s fixed, H&E stain, 400× magnification). C, weights of testes and epididymides after OSU-HDAC42 treatment from 6 to 10 (left) or 24 (right) wk of age, and 5 wk or 6 mo after drug withdrawal. OSU-HDAC42 significantly reduced testis and epididymis weights in treated TRAMP mice. Tissue weights were restored in similarly treated wild-type mice after withdrawal of the drug. Columns, mean; bars, SD (n = 15 and 23 TRAMP mice at 10 and 24 wk, respectively; n = 5 wild-type mice in the recovery groups). **, P < 0.01. D, left, Western blot analysis of TAg expression in the prostates of wild-type mice and TRAMP mice treated from 6 to 10 or 24 wk of age. Prostatic TAg expression is retained in drug-treated TRAMP mice. Right, serum total testosterone levels in control and treated TRAMP mice. Testosterone levels were unaltered by drug treatment. Testosterone levels were measured using an enzyme immunoassay kit (Cayman Chemical Company) according to the manufacturer’s recommendations. Columns, mean; bars, SD (n = 5). T, testosterone.
in OSU-HDAC42 diet–fed and control diet–fed mice both at 10 and 24 weeks of age. Moreover, serum testosterone levels in drug-treated mice were not significantly different from those in controls, refuting the involvement of altered TAg expression or androgen production in the suppressive effects of OSU-HDAC42 on tumorigenesis (Fig. 5D).

Discussion

The intensity of efforts to develop HDAC inhibitors for cancer therapy is reflected by the recent FDA approval of vorinostat (10) and the current use of at least 10 different HDAC inhibitors in >80 clinical cancer trials.5 Far fewer studies, however, address the chemopreventive activities of these agents (12). The features of HDAC inhibitors that underlie their preclinical and clinical successes to date as cancer therapeutic agents, such as their broad spectrum of activities against regulators of cellular differentiation, cell cycle, and apoptosis, as well as their differential toxicity (8, 39) for transformed cells, also predict favorably for their chemopreventive activity and are especially fitting for the molecularly heterogeneous and initially latent disease of prostate cancer. However, lack of full understanding of HDAC biology precludes the complete mechanistic characterization of HDAC inhibitors and hinders the prediction of drug-associated toxicity (18). Consequently, this study is aimed at providing insight into both the chemopreventive efficacy and toxicity of chronic therapy of OSU-HDAC42 by examining its in vivo effect on tumorigenic and phenotypic end points in TRAMP mice.

To establish the antitumor efficacy of the dietary administration of OSU-HDAC42, the in vivo effects of dietary delivery were compared with that of gavage administration in PC-3 tumor-bearing mice. Despite the presumed differences in the pharmacokinetic behaviors of OSU-HDAC42 given by these two methods of drug delivery, the effect on end points of efficacy (PC-3 tumor volume) and toxicity (testis/epididymis weight, hematologic variables) were, nevertheless, equivalent. This finding established the feasibility of dietary administration of OSU-HDAC42 and suggests a desirable pharmacokinetic feature of the drug, which provides flexibility in its delivery method in cancer treatment.

Targeting PIN as a method to control prostate cancer was recently validated by the association of the cancer preventive activity of finasteride with reduced risk of PIN in the Prostate Cancer Prevention Trial (35). Similarly, in our study, the suppression of PIN in TRAMP mice by OSU-HDAC42 correlated with a marked retardation of prostate tumorigenesis. It is noteworthy that in 24-week-old TRAMP mice OSU-HDAC42 completely inhibited the occurrence of poorly differentiated carcinoma and suppressed the relative UGT weight to that of 10-week-old control TRAMP mice. To our knowledge, this is the most potent chemopreventive effect achieved by a small molecule agent in TRAMP mice with documentation of the effects on body weight. The class I HDAC inhibitor MS-275 induced much smaller reductions in macroscopic tumor incidence (7 of 10 controls versus 5 of 11 drug-treated animals) and UGT weights of TRAMP mice treated over an age interval and with a drug dose similar to those in our study (from 5–7 to 27 weeks of age and 20 mg/kg/d; ref. 40). Considering that OSU-HDAC42 affects both classes I and II HDAC substrates (20) and that the clinical significance of improving HDAC isoform selectivity is undetermined (41), these findings support a role for broad spectrum HDAC inhibitors in prostate cancer chemoprevention.

The high incidence of adenomas in OSU-HDAC42–treated TRAMP mice is noteworthy. The adenoma phenotype is not included in the spectrum of lesions described by the founders of the model (25) and is rarely reported in TRAMP mice by other investigators. Whereas the mechanistic basis for adenoma formation in response to drug treatment is uncertain, the prodifferentiation effects attributed to HDAC inhibitors are potentially involved. Consistent with this notion are the changes in E-cadherin and synaptophysin expression observed in representative prostate tissue from control and OSU-HDAC42–treated TRAMP mice at 24 weeks. Although these adenomas were limited to the DPs, the reported location of phyllodes-like tumors in TRAMP mice (25), their morphology was distinctly different from phyllodes-like lesions in that they contained a scant stromal compartment. The single phyllodes-like tumor detected in this study occurred concomitantly with an adenoma in the same DP of a drug-treated mouse. Whereas these adenomas could be interpreted to represent a drug-induced alteration of phyllodes-like tumor growth, the coexistence of these lesions suggests they were unrelated.

The exact cause of OSU-HDAC42–induced testicular degeneration remains undetermined; however, based on unchanged serum levels of testosterone and TAg expression in treated TRAMP mice, this toxicity does not seem to involve disrupted androgen production. This is an important point because carcinogenesis in the TRAMP model is initially dependent on androgen to induce TAg expression through the probasin promoter. Thus, unlike flutamide, which decreased prostate lesion development in TRAMP mice in association with its antiandrogen activity and suppression of TAg (42), the potent chemopreventive effects observed in our study are attributed to the direct effects of OSU-HDAC42 on the prostate. The sparing of spermatogonia in the degenerative testes of treated mice correlates well with the reversible nature of the lesion. Because the leptotene-zygote spermatocytes are the most progressed cell types of the spermatogenic cycle remaining in drug-treated testis, OSU-HDAC42 likely disrupts the successional stage of meiotic prophase, the pachytene-diplotene primary spermatocytes.

The lack of testicular degeneration in vorinostat-treated mice in our pilot study shows that this toxicity is not common to all agents in this class. To our knowledge, the only other report of HDAC inhibitor–associated testicular degeneration involved trichostatin-A (TSA), which was also concluded to target spermatocytes (43). Although a direct link to testicular HDAC inhibition has not been shown with TSA or OSU-HDAC42, this association is plausible given the critical role of HDACs in the control of chromatin condensation during spermatogenesis (44). Differences in HDAC isoform selectivity could play a role in this toxicity given the high expression of HDAC6, against which OSU-HDAC42 and TSA have potent inhibitory activity (20, 45), in normal testis compared with other tissues (46). In any event, it should be noted that OSU-HDAC42 has not been evaluated clinically yet, thus its effect on human testis is unknown and a conclusion regarding the risk–efficacy ratio for this toxicity in man would be speculative at this time. In fact, the consistent, potent, and reversible effect of OSU-HDAC42 on the testes, with sparing of major organ histology and weight, may warrant its evaluation for the treatment of testicular cancer.

5 http://www.clinicaltrials.gov
The transient decrease in circulating blood cells that occurred in OSU-HDAC42–treated mice without effects on bone marrow cellularity is different from the myelosuppression observed with cytotoxic agents currently in clinical use and, as is suspected with other HDAC inhibitors, suggests a mechanism other than toxicity to early hematopoietic precursors (47). Compensatory EMH in the spleens of OSU-HDAC42–treated mice may explain the absence of leukopenia after 18 weeks of treatment. The significance of these hematologic alterations, which must be interpreted in light of their magnitude and in conjunction with all pathology variables and in-life observations (48), requires further preclinical evaluation and dose titration to assess potential implications for human patients. Importantly, no lesions were detected in major organs, either by histologic assessment or by organ weight evaluation (Supplementary Table S2), including heart, liver, kidney, lung, adrenal gland, pituitary gland, and brain, that correlated with the hematologic abnormalities.

In summary, OSU-HDAC42 suppressed the severity of PIN and completely blocked the progression of PIN to poorly differentiated carcinoma in the TRAMP model. The tumor suppressive effects were associated with intraprostatic modulation of HDAC and targets regulating cancer cell survival and differentiation. These results suggest clinical value in incorporating OSU-HDAC42 into the management regimen of patients with PIN. In addition to effects of drug treatment on the prostate, we partially characterized reversible testicular degeneration and identified hematologic alterations that should be considered for monitoring in the future preclinical and clinical use of this and other HDAC inhibitors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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We dedicate this manuscript to coauthor Russell Klein, who passed away on December 1, 2006, after a year-long battle with acute leukemia. Russell’s legacy will live on from the lives touched and contributions made to the Molecular Carcinogenesis and Chemoprevention Program of The Ohio State University Comprehensive Cancer Center.

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OSU-HDAC42, a Histone Deacetylase Inhibitor, Blocks Prostate Tumor Progression in the Transgenic Adenocarcinoma of the Mouse Prostate Model


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