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Histone Deacetylase Inhibitor: Antineoplastic Agent and Radiation Modulator

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Abstract

Inhibitors of histone deacetylases (HDACs) have emerged as a new class of anticancer agents based on their actions in cancer cell growth and cell cycle arrest, terminal differentiation, and apoptosis. Previously, we rationally designed and developed a new class of hydroxamide- and mercaptoacetamide-bearing HDAC inhibitors. A subset of these inhibitors exhibited chemo-radiation sensitizing properties in various human cancer cells. Furthermore, some HDAC inhibitors protected normal cells from radiation-induced damage and extended the survival of mice following total body exposure to lethal dose radiation. Pathological analyses revealed that intestinal and bone marrow cellularities recovered significantly from radiation-induced damage by structural compartments restoration, suggesting the mechanism of action of these HDAC inhibitors. These findings support the hypothesis that epigenetic regulation may play a crucial role in the functional recovery of normal tissues from radiation injuries.

The epigenetic regulation governs gene expression, in part, through the balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities. HATs acetylate lysine groups at the amino terminal tails of nuclear histones to neutralize positive charges on the histones, yielding a more open, transcriptionally active chromatin structure [1]. By contrast, HDAC family members deacetylate lysine residues on histones and induce transcription repression through chromatin condensation. Increased acetylation of histones and nonhistone proteins leads to changes in the chromatin architecture and accessibility for key cellular proteins to specific target sites [2, 3]. As such, a shift in the relative activities of these enzymes profoundly influences biological processes, including DNA repair, replication, cell cycle checkpoint activation, and cellular differentiation [4].

14.1 Histone Deacetylase Family Members

HDACs are initially identified as components of large multiprotein complexes that bind to promoters and repress transcription, and are found in the nuclear and cytoplasmic compartments [5, 6]. Eleven human HDACs have been identified and divided into four classes based on structure, sequence homology, and domain organization. Class I consists of HDACs 1, 2, 3, and 8, which are predominantly nuclear and play roles in cell proliferation [5]. Class II HDACs are further subdivided into IIa (HDACs 4, 5, 7, and 9) and IIb (HDAC6 and 10) [7]. These enzymes are characterized by a large NH₂-terminal domain or a second catalytic site and their expression is more restricted, suggesting roles in cellular differentiation and development [8]. Class III enzymes include the SIRT_s (sirtuins), and are NAD-dependent deacetylases [9], which are not inhibited by TSA or other hydroxamates.

HDAC 11 is characterized as class IV, based on a phylogenetic analysis, and whose function is least known [10].

14.2 Structural Classifications of HDAC Inhibitors (HDACIs)

Inhibitors of HDACs have been discovered and developed as promising anticancer drugs [11]. On the basis of structural divergence, HDAC inhibitors are classified: (1) short-chain fatty acids (i.e., sodium phenyl butyrate, valproic acid, AN-9), (2) hydroxamic acids (i.e., suberoylanilide hydroxamic acid (SAHA; vorinostat), oxamflatin, trichostatin A (TSA), *m*-carboxycinnamic acid bis-hydroxamide (CBHA), LBH-589, LAQ-824, PCI-24781), (3) cyclic peptides (i.e., FR901228 (depsipeptide; romidepsin), apicidin, cyclic hydroxamic acid-containing peptides (CHAPS), trapoxin), (4) benzamides (i.e., MS-275, CI-994), (5) ketones (i.e., trifluoromethyl ketone), and (6) miscellaneous (i.e., MGCD-0103). A subset of HDACIs is reportedly as pan-HDAC inhibitors, such as TSA and SAHA, while MS-275 and depsipeptide confer isomer or/and class-specificities. Some of which are currently in clinical trials [12], and two drugs, vorinostat (Zolinza®) and romidepsin (Istodax®), have recently been approved by FDA for the treatment of cutaneous T-cell lymphoma (CTCL) in patients ([13], www.hhs.gov).

14.3 Development of Second-Generation HDACIs

Despite a generally acceptable toxicity profile for HDACIs, the lack of target specificity and the presence of the toxicophore hydroxamide group may still cause unpredictable toxicity problems. To improve physicochemical and pharmacological properties of first-generation HDACIs, we rationally designed and developed several new classes of HDACIs, including ligands bearing either a hydroxamate or mercaptoacetamide group [14, 15]. A subset of these candidates exhibited 50% HDAC inhibition activity within nanomolar ranges *in vitro* and radiosensitizing effects on various cancer cells [14, 15]. The candidate inhibitors potently repressed the growth of cancer cells: the GI₅₀s for the hydroxamates ranged from 0.1 to >60 μM, while the mercaptoacetamides revealed weaker growth inhibitory activity against cancer cell lines. Table 14.1 presents the data demonstrating that the hydroxamates conferred potent cytotoxicity, while the mercaptoacetamides revealed less against all three cancer cell lines. However, normal human fibroblasts (NHP-5) and human primary skin fibroblasts (Hs-68) were found to be significantly resistant to these HDACIs, supporting the notion that candidate HDAC inhibitors preferentially suppress cancer cell proliferation.

14.4 Cellular Target Specificity of HDACIs

Given that 11 HDAC isomers are present in mammalian cells, whether isomer-specific HDACIs are better drugs has been an outstanding question and is actively in ongoing investigation. In general, the determination of acetylated histone and α-tubulin levels has been used as a measure of HDACI efficacy *in vitro* and *in vivo*. Pan-HDACIs, such as SAHA and TSA, appear to target both histones H3/H4 and α-tubulin evidenced by their enhanced acetylation levels, in a dose-dependent manner. In contrast, other HDACIs, such as trapoxin B, sodium butyrate, and FK-228, do not exhibit these characteristics, suggesting their selectivity at the cellular level [17]. Others have reported that depsipeptide exerts modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells [18]. Valproic acid, an anti-epileptic agent, has shown to be involved in the proteolysis of HDAC2 [19]. Hyperacetylations of α-tubulin and Hsp90 have also been tightly linked to class II HDAC6 inhibition. Our data, as shown in Fig. 14.1, demonstrate that mercaptoacetamide-based HDACI 6MAQH preferentially enhances acetylation of α-tubulin while hydroxamate HDACIs, SAHA and H6CAHA, enhance the acetylation levels of both histone H4 and α-tubulin in a dose-dependent manner, suggesting 6MAQH as a class II HDAC isomer-specific inhibitor. Therefore, understanding of the roles of various HDAC isoforms in cell

processes and identifying HDACI-specific biomarkers in diseases may contribute to strategies for the identification of promising second-generation inhibitors that exhibit target specificity and that offer clinical relevance.

14.5 HDACs, DNA Damage Repair, and Radiation Sensitivity

Accumulated studies have shown that a particular subset of HDACs induces radiosensitivity of various human cancer cells [14, 20–24]. Consistent with these findings, several recent reports have provided supporting evidence of specific HDAC isoforms' roles in cell cycle regulation and intrinsic radiation sensitivity [25–29].

A significant line of evidence has demonstrated that a subset of HDAC family members plays a critical role in DNA damage response and repair [27, 30–32] and that post-translational modifications (e.g., acetylation) of histones or nonhistone proteins flanking DSBs affect accessibility of repair and signaling proteins to the damaged regions of DNA [33, 34]. Moreover, changes in the chromatin architecture by HDACI have shown to enhance the activation of nonhistone proteins, including p53, pRB, and transcriptional factors that are involved in cell cycle regulation and affect cell proliferation and apoptosis, processes that are frequently aberrant in cancers [11, 35]. With their intrinsic anticancer properties, a subset of HDAC inhibitors has exhibited radiation sensitizing properties in certain cancer cell lines and in preclinical models [20–24, 36, 37]. However, underlying mechanisms are not fully understood.

Previously, we have shown that an HDAC inhibitor, trichostatin A (TSA), increased the amount of radiation-induced DNA damage and slowed the repair kinetics [31]. Gene expression profiling also revealed that a majority of genes that control cell cycle, DNA replication, and damage repair processes were down-regulated following TSA exposure. HDACI treatment modulated the radiation-induced DNA damage repair process, in part, by suppressing *BRCA1* gene expression [31]. Further studies have demonstrated prolonged appearance of γ H2AX and Rad51 foci and suppression of DNA damage repair genes (*ATM*, *Brca1*, and *Brca2*). Functions of damage sensing and repair proteins, including ATM, MRE11, γ -H2AX, and 53BP1, are also affected by changes in chromatin structure conformation [38–40].

A sensor of DNA damage, ATM has been shown to interact with HDAC1, to play a role with HDAC4 in the DNA damage response pathway, and to be associated with ionizing radiation-induced changes in chromatin structure [25, 28]. Inactivation of HDAC3 affects S phase progression and enhances DNA damage [26]. HDAC4 co-localizes with 53BP1 (a protein which participates in the phosphorylation of p53 and Chk2 and in the maintenance of S and G₂ cell cycle checkpoints) in DNA damage-induced nuclear foci [25]. Finally, other studies have suggested that butyrates, TSA, and inhibitory HDAC RNAs are capable of sensitizing cancer cells to ionizing radiation [27, 30–32, 41].

14.6 HDACI and Radioprotection In Vitro

One of the hallmarks of HDACI action is to preferentially sensitize actively proliferating cancer cells [42–45]. Normal cells are considerably more resistant to HDAC inhibitors than are tumor cells [45]. To determine underlying mechanisms of biological discrepancy between cancer and normal cells, we examined the synergistic effect of HDACI on DNA damage response by scoring phosphorylated H2AX (γ H2AX) foci, one of the first events occurring at DNA double-strand breaks (DSB). The data demonstrated that the foci formation in cancer cells (PC3, LNCaP, and DU145) was markedly increased within 0.5 h and gradually decreased by 3 h after irradiation (5 Gy) (Fig. 14.2). HDAC inhibitor treatment alone resulted in the accumulation of γ H2AX foci formation. However, after

pretreatment with H6CAHA for 16 h followed by irradiation (5 Gy), γ H2AX foci/cell growth increased significantly and sustained these levels up to 6 h and persisted for at least 24 h. This trend was not observed in normal cells (data not shown). These results indicate that disruption of the radiation-induced DSB damage repair mechanism may account for H6CAHA-induced radiosensitization of cancer cells and also indicate that H6CAHA may have the potential to facilitate DNA damage repair in normal cells.

14.7 HDACI and Radioprotection In Vivo

Given that the ability to selectively kill cancer cells while limiting damage of normal cells is the ultimate therapeutic goal and that HDACIs (phenylbutyrate, trichostatin A, and valproic acid) could suppress cutaneous radiation syndrome [42, 43], understanding the mechanisms of cancer cell radiation sensitization and normal cell protection by HDACIs is important.

To address how the candidate HDACIs affect normal cells in response to ionizing radiation in vivo, C57BL/6 mice were treated with an i.p. subtoxic dose (4 mg/kg) of either hydroxamate H6CAHA or mercaptoacetamide 6MAQH, 4 h prior to total-body irradiated (TBI) with 15 Gy of gamma radiation. The survival rate and body weight were monitored daily. The data demonstrated that 100% of animals undergoing TBI died within 6–7 days. The loss of body weight was evident, possibly indicating intestinal damage. However, HDACI treatment extended the survival of animals; 40% of animals treated with hydroxamide H6CAHA or mercaptoacetamide 6MAQH survived until the end of the study at 25 days. The data also showed that the duration of survival of animals pretreated with hydroxamate H6CAHA (median survival: 14 days) extended further than those treated with mercaptoacetamide 6MAQH (median survival: 9 days) (Fig. 14.3). These animals eventually gained weight and continued to survive until termination of the experiments.

To understand underlying mechanisms, histological analyses were performed using various tissues, including the pelvic bone marrow (BM) and the intestine. The data demonstrated that HDACI pretreatment resulted in less BM cell loss within 2 days compared with treatment with IR alone (Fig. 14.4). Structural recovery accompanied by an increase in small intestine crypts with villus height was also interpreted as an indicator of radioprotection (Fig. 14.5).

Although the underlying mechanisms responsible for survival remains unclear, these results suggest that the candidate HDACIs protect mice from BM and intestinal injuries after total-body irradiation (TBI), supporting the clinical potential of HDACI in protecting the damage of normal cells/tissues during radiation treatment. It is attempted to speculate that candidate HDAC inhibitors may trigger the activation of BM progenitors and promote their infiltration into the damaged sites and that suppression of cytokines by candidate compounds may contribute to reduced inflammation and/or cell death rates, thereby facilitating recovery from radiation-induced injuries.

14.8 Conclusion

Radiation-induced acute and late injuries often cause dysfunction of organs and generate secondary effects on functional impairment [46, 47]. Limitations and efficacy of radiation therapy in cancer treatment are contingent upon tolerances of normal tissues encompassed in the irradiated fields. Our novel and structurally distinct HDACIs, hydroxamate H6CAHA and mercaptoacetamide 6MAQH, exhibited modulating effects on radiation responses by which preferentially killing cancer cells in vitro and in vivo, and enhancing the survival rate of mice following lethal doses of radiation. The data suggest that significant recovery of intestinal and BM cellularities and restoring structural compartments from radiation-induced damage is a mechanism of action of these HDAC inhibitors. Therefore, the use of HDAC

inhibitors for radiosensitizing cancer cells and radioprotection of normal tissues identifies an entirely new category of drugs with clinical translation potential. Further studies are warranted for its precise evaluation in which these candidate compounds can be advanced as a treatment agent for normal cell protection from radiation-induced injury.

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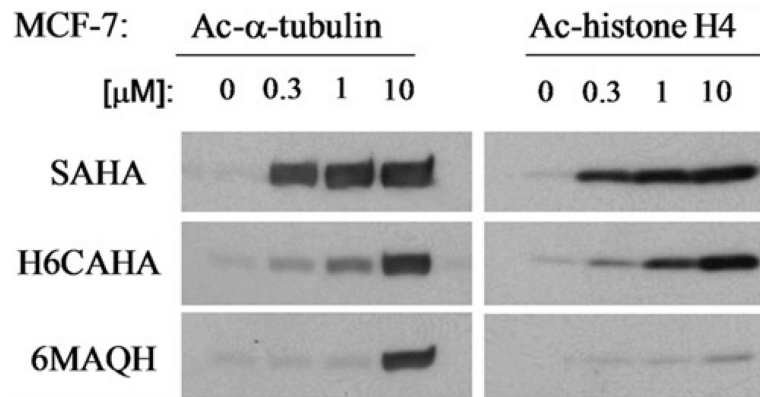


Fig. 14.1.

Dose-dependent and differential effects of hydroxamate H6CAHA and mercaptoacetamide 6MAQH on histone H4 and α -tubulin acetylation levels in MCF7 cells were analyzed after 4-h treatment with indicated concentrations of HDAC inhibitors. Cells lysates were prepared in a lysis buffer containing 20-mM Tris-HCl (pH 7.4), 2-mM EDTA (pH 7.4), 2-mM EGTA (pH 7.4), 6-mM mercaptoethanol, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1% NP40. The samples were subjected to SDS-polyacrylamide gel electrophoresis. The indicated proteins were probed with a polyclonal rabbit antibody against acetylated histone H4 or α -tubulin (Upstate, Inc). The immune-reactive bands were detected by enhanced chemiluminescence (Amersham Biosciences)

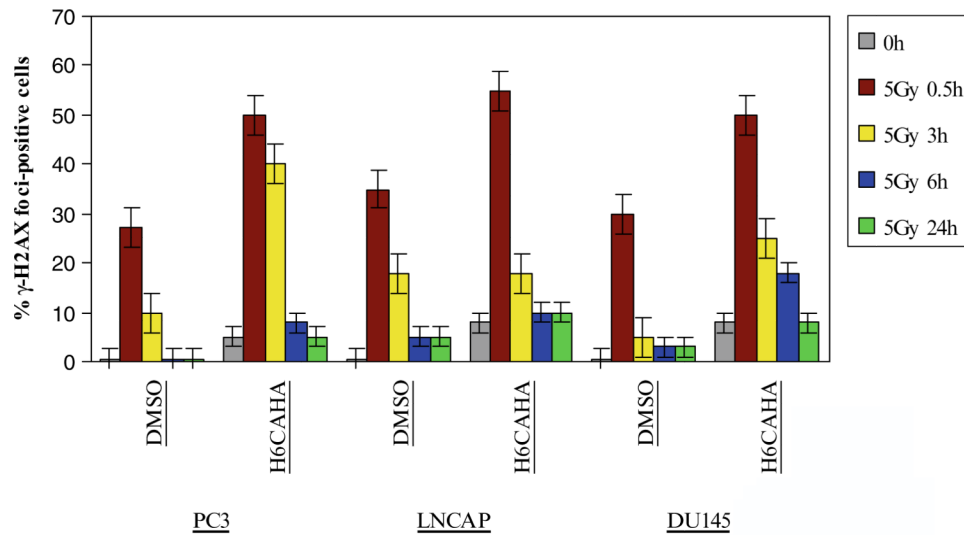


Fig. 14.2.

Effect of H6CAHA on radiation-induced γ H2AX foci; PC3, LNCaP, and DU145 cells were incubated with vehicle or 1- μ M H6CAHA for 16 h and then exposed to 5 Gy of γ -radiation. The 0-h samples were not exposed to radiation. γ H2AX foci were evaluated in 75 nuclei per treatment per experiment. Columns represent mean values from three independent experiments; *bars* represent SD. After staining, foci were visualized by Olympus FV300 equipped with FluoView software. Statistical comparisons between treatment groups were made using GraphPad Prism 4.0 software by Student's *t*-test. A probability value of $p < 0.05$ was considered to be significant

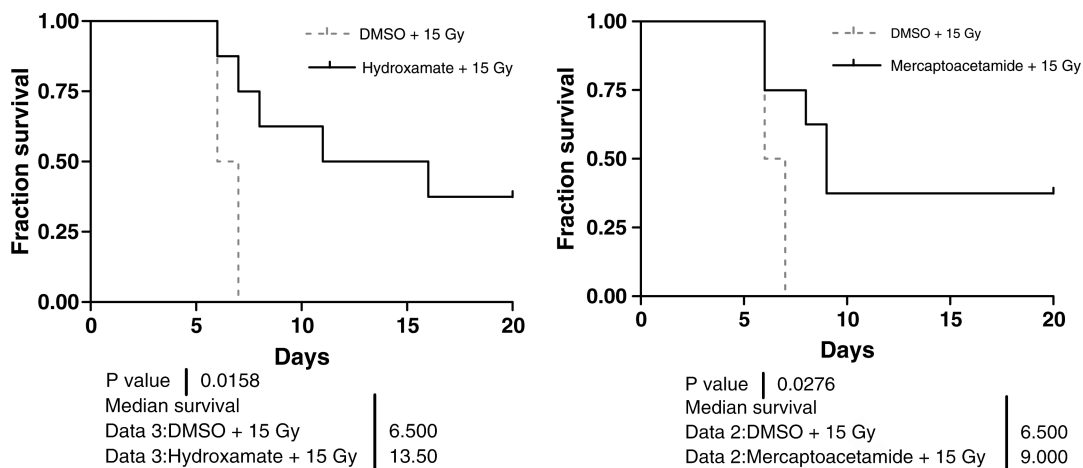


Fig. 14.3.

The effects of each potential HDAC inhibitor, hydroxamate (H6CAHA) and mercaptoacetamide (6MAQH), on survival of mice undergoing total body irradiation of 15 Gy gamma. Each cohort (10 mice per group) received i.p. administered HDACI (4 mg/kg body weight) 4 h prior to irradiation. Survivals of animals were monitored and the body weights were measured daily for 25 days until the end of experiments. C57BL/6 mice (18–22 g) were treated with an intraperitoneal dose (4 mg/kg) of hydroxamate or mercaptoacetamide, 4 h prior to total-body irradiation (TBI). The animal protocol was reviewed and approved by the Georgetown University Animal Welfare Committee. The body weights of control and treated mice were measured at weekly intervals. Total body irradiation (TBI) was performed using a Mark-30 irradiator with a ^{137}Cs source at a fixed dose rate of 2.71 Gy/min. The Kaplan-Meier method was used to analyze survival data. All statistical analyses were performed on relative survival data (percentage change from initial survival). Student's *t*-tests were performed to determine if the differences between the treatments were significant. $p < 0.05$ was considered statistically significant

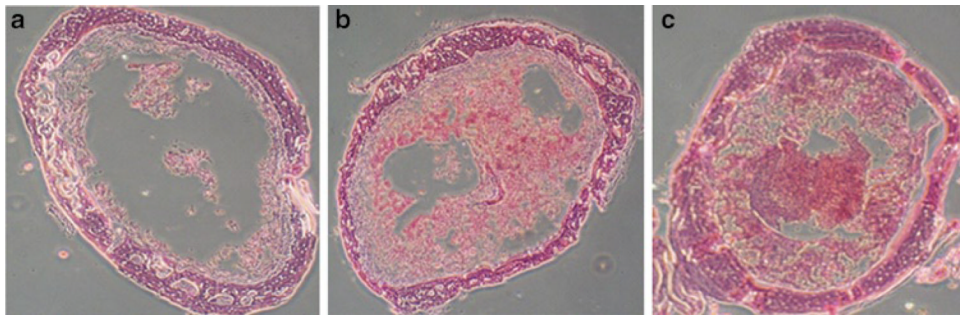


Fig. 14.4. Mouse bone marrow (BM) 2 days after exposure to 15 Gy of Cs-137 total body irradiation (IR). Femur cross sections: (a) DMSO (control); (b) 4 mg/kg of mercaptoacetamide 6MAQH; (c) 4 mg/kg of hydroxamate H6CAHA

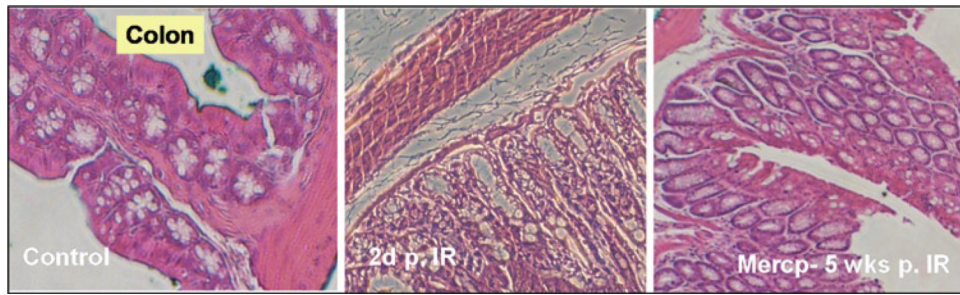


Fig. 14.5. Mouse intestine (colon) after exposure to 15 Gy of Cs-137 total body irradiation (IR). *Left:* DMSO control; *Middle:* 2 days post-IR; *Right:* merceptoacetamide 6MAQH, 5 weeks post-IR

Table 14.1

Effects of hydroxamate (H6CAHA) and mercaptoacetamide (6MAQH) HDAC inhibitors on pan-HDACs and the growth of cancer and normal cells

Compound	50% pan-HDAC activity inhibition (nM)	Cell proliferation (GI ₅₀ μ M)				
		Cancer cells			Normal cells	
		PC-3	SQ-20B	MCF-7	NHP-5	Hs-68
H6CAHA	176	1.77	2.88	3.98	>300	>500
6MAQH	44	40	30	23	>500	>500

Note: Cell lines were obtained from the Tissue Culture Shared Resources of the Lombardi Comprehensive Cancer Center, Georgetown University Medical Center. PC-3 is a prostate cancer cell line; SQ-20B is a head and neck squamous carcinoma cell line; MCF-7 is a breast cancer cell line; NHP-5 and Hs-68 are nonmalignant human epithelial cell lines. Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after a 48-h incubation period, as previously described [16]. Growth inhibitory concentrations at 50% (GI₅₀) were calculated graphically and the mean GI₅₀ values were obtained. Results are the mean of at least three determinations. Statistical comparisons between treatment groups were made using GraphPad Prism 4.0 software