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EDITORIAL

Deacetylase inhibitors - focus on non-histone targets and effects

Matthias Ocker

Matthias Ocker, Institute for Surgical Research, Philipps University Marburg, Baldingerstrasse, 35033 Marburg, Germany
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Supported by Supported by a Research Grant of the University Medical Center Giessen and Marburg
Correspondence to: Matthias Ocker, MD, Professor of Medicine, Director, Institute for Surgical Research, Philipps University Marburg, Baldingerstrasse, 35033 Marburg, Germany. ocker@staff.uni-marburg.de
Telephone: +49-6421-5868930 Fax: +49-6421-5868926
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Abstract

Inhibitors of protein deacetylases have recently been established as a novel therapeutic principle for several human diseases, including cancer. The original notion of the mechanism of action of these compounds focused on the epigenetic control of transcriptional processes, especially of tumor suppressor genes, by interfering with the acetylation status of nuclear histone proteins, hence the name histone deacetylase inhibitors was coined. Yet, this view could not explain the high specificity for tumor cells and recent evidence now suggests that non-histone proteins represent major targets for protein deacetylase inhibitors and that the post-translational modification of the acetylome is involved in various cellular processes of differentiation, survival and cell death induction.

Key words: Epigenetics; Histone deacetylase inhibitors; Histone code; Posttranslational modifications; Unfolded protein response

Peer reviewers: Sinisa Dovat, MD, DSc, Assistant Professor, Director, Molecular Oncology Research, University of Wisconsin - Madison, Department of Pediatrics, Division of Pediatric Hematology/Oncology, 4137 Wisconsin Institutes for Medical Research, 1111 Highland Ave., Madison, WI 53705-2275, United States; Jianping Ye, MD, Professor of Molecular Biology, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808, United States

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INTRODUCTION

Histones were historically viewed as structural proteins allowing the chromatin to be folded into the nucleus. The discovery of post-translational modifications like methylation and acetylation in the 1960s proposed functions in addition to mere scaffolding of DNA[1,2]. Acetylation and deacetylation are achieved by two groups of highly conserved enzyme families termed histone acetyl transferases (HAT) and histone deacetylases (HDAC) because histones were their first identified targets.

HISTONE ACETYL TRANSFERASES

HAT comprise an evolutionary highly conserved group of enzymes. The first HAT was identified in yeast as Hat1[3] and so far more than 30 different HATs with known substrate specificity have been described in mammals[4-6]. Two general classes of HATs have been described: A-type HATs which mainly regulate transcriptional processes and show a nuclear localization and B-type HATs which are predominantly expressed in the cytoplasm[7,8].

HATs usually transfer acetyl groups to ε-amino groups of lysine residues in basic core histone proteins which alters the DNA-histone or DNA-protein interaction due to altered electrochemical properties between positively charged histones and the negatively charged DNA backbone[9,10]. This and additional steric effects of acetylated

lysine residues render the chromatin in an "open" conformation and thus facilitates the binding of transcription factors[11]. These post-translational modifications were viewed as a key regulatory process for gene transcription and hence the term "histone code" was coined[12-14]. As the effect of HAT and HDAC are rather broad, the concept of a code that specifically regulates target gene expression has been weakened and data from gene array experiments show that only 2%-10% of all genes are regulated by this mechanism[15,16].

HISTONE DEACETYLASES

Today, four different classes with at least 18 subtypes of HDAC have been described[11,17,18]: class I (Rpd3-like) represents the ubiquitously expressed zinc-dependent isotypes HDAC1, 2, 3 and 8. These isotypes are usually localized in the nucleus and act as transcriptional corepressors. Class Ⅱ (Had1-like), consisting of HDAC4, 5, 6, 7, 9 and 10, shows a tissue-specific distribution and is localized in the nucleus and the cytoplasm, although histone proteins also seem to represent the major target. Class Ⅲ (sirtuins) represents the zinc-independent enzymes SIRT1 to SIRT7 that have been associated with cell proliferation and cell cycle control. HDAC11 has homology to the class I HDACs but shows a more restricted tissue distribution and also has a cytoplasmic localization and is therefore considered a distinct 4th class of HDAC[19,20]. Interestingly, HDAC6 and HDAC10 (also designated as class Ⅱb) have two catalytic sites and show a mainly cytoplasmic localization.

The overexpression of HDAC isoenzymes has also been linked to a variety of human cancer diseases[17], e.g. HDAC1 in gastric[21] or breast cancer[22], HDAC3 in colon cancer[23] and HDAC6 also in breast cancer[24], without a complete understanding of whether this overexpression is a precondition of the malignant phenotype or a consequence of a general process of gene dysregulation. Little is known about the different functions of the HDAC isotypes in the investigated tissues.

INHIBITORS OF HISTONE DEACETYLASES

Several chemically distinct histone deacetylase inhibitors (HDACi) have been developed and are currently undergoing clinical trials[11,18]. So far, only the hydroxamate vorinostat [suberoylanilide hydroxamic acid (SAHA)] has been approved for clinical use in patients with cutaneous T-cell lymphoma[25]. Generally, hydroxamates like vorinostat, panobinostat (LBH589), trichostatin A or belinostat (PXD101) are unspecific HDACi with activity against classes Ⅰ, Ⅱa, Ⅱb and Ⅳ of HDAC[26]. Other compounds specifically inhibit class I HDACs, e.g. the benzamide MS-275 or the cyclic tetrapeptide depsipeptide, or class I and Ⅱa HDACs, e.g. the short-chain fatty acids valproic acid or butyrate[26,27]. As the various roles of HDAC isotypes are still unclear it is also under debate whether isotype or class specific HDACi should be preferred over the broad-spectrum inhibitors. The selectivity of the compounds has also been demonstrated by a divergent pattern of acetylation of α-tubulin, histone H3 and histone H4, which indicates that these proteins may not be suitable biomarkers for the clinical use of all HDACi[26,28]. Except for the HDAC6-specific compound tubacin[29], all classes of HDACi lead to a G_1/S-phase arrest of the cell cycle which is commonly associated with an increased expression of the endogenous cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$[17,30,31]. Although $p21^{cip1/waf1}$ has been shown to be a transcriptional target of p53[32-34], HDACi mediated induction of $p21^{cip1/waf1}$ can occur by p53-dependent and -independent mechanisms[35,36], and our own results showed that the anti-tumor effect of HDACi is independent of p53 but is facilitated by the absence or inhibition of $p21^{cip1/waf1}$[37-39].

CLINICAL APPLICATION AND EXPERIENCE WITH HDACI

Today, only the pan-deacetylase inhibitor SAHA (vorinostat) has been approved for clinical use in cutaneous T-cell lymphoma by the FDA[40]. Other HDACi are currently undergoing intensive clinical investigations in various human tumor entities, with most trials focusing on hematologic indications such as acute or chronic leukemias, myelodysplastic syndrome or lymphomas[41,42]. Although the currently available results are promising with regard to their overall response rates (usually around 30%), no long-term experience with these compounds has been reported. From a clinical point of view, it is also unclear whether the more specific class I and Ⅱ inhibitors like MS-275 or the pan-deacetylase inhibitors like SAHA or panobinostat provide better treatment responses. Interestingly, the toxicity profiles of HDACi are comparable, mainly gastrointestinal side effects (diarrhea), myelosuppression and cardiac QT prolongation, over the different types of HDACi. Most HDACi have a rather short half-life in plasma (2-8 h) and undergo hepatic metabolization with subsequent intestinal excretion[43-48]. Yet, the effect of HDACi on histone H3 acetylation in peripheral blood mononuclear cells is stable for approximately 10 h after oral dosing[43]. This was initially considered a suitable biomarker for the efficacy of HDACi in tumors but several studies were unable to establish a correlation between peripheral H3 acetylation and tumor target effects, probably due to the numerous non-histone targets of HDACi to be discussed later[41,42].

APOPTOSIS INDUCTION BY HDACI

HDACi have been demonstrated to be potent inducers of apoptotic cell death in human tumor cells and also show strong effects in various animal models and in clinical trials. Interestingly, these compounds show a high specificity for tumor cells with a so far unknown mechanism of action. This observation (along with the low number of genes affected by HDACi) is in contrast

to the established view of these compounds as general modulators of chromatin and transcription.

It was demonstrated that HDACi induce apoptotic cell death by activating both, the extrinsic (TRAIL-mediated) and the intrinsic (mitochondria-related) pathway of apoptosis, e.g. by upregulation of death receptors (hDR4, hDR5) or suppression of bcl-2 family proteins[38,49-56]. Although most of these effects are also mediated by the transcriptional control of hyperacetylated p53 or histones[57], other pro-apoptotic effects of HDACi, e.g. the generation of reactive oxygen species, also contribute to this effect[58-60] and are currently not understood at the molecular level.

Additional anti-cancer effects of HDACi include a synergistic enhancement of other cytostatic agents, including gemcitabine[61], 5-fluorouracil and irinotecan[62], but also targeted agents like imatinib[63], bortezomib[64], sorafenib[65,66] or antibodies like trastuzumab[67]. *In vivo*, HDACi also inhibit angiogenesis by decreasing the expression of pro-angiogenic factors like vascular endothelial growth factor and hypoxia inducible factor-1α (HIF-1α) or inducing endothelial cell death[39,68-71]. These effects are also closely associated with an inhibition of invasion and metastasis by HDACi[72,73].

HDACi have also been associated with a variety of immunomodulatory effects, e.g. by hyperacetylation of STAT1, STAT3 or NF-κB and a subsequent modulation of downstream inflammatory pathways[74-80]. Besides these gene regulatory effects, HDACi can also exert immunologic effects by affecting the acetylation status of regular cellular proteins which are then presented *via* major histocompatibility complexes Ⅰ and Ⅱ.

NON-HISTONE TARGETS AND EFFECTS OF DEACETYLASE INHIBITORS

Post-translational modifications of proteins, including phosphorylation, ubiquitination, methylation, acetylation and other processes, are important regulators of protein function and homeostasis in eukaryotic cells[81]. Although protein acetylation at lysine residues was discovered in the 1960s[2], analysis of the acetylome was hampered due to technical reasons[82,83]. Two recent studies have now provided new insights into the acetylome as well as the effects of different HDACi, and showed that various cellular networks, e.g. RNA splicing, DNA damage repair, cell cycle control, nuclear transport, actin remodeling, ribosome and chaperone function are intensively affected by changes in protein acetylation[84,85] which were previously postulated on a theoretical basis[86]. These studies also showed that > 20% of mitochondrial proteins are acetylated, which provides a functional connection between the localization of the class Ⅲ HDAC to mitochondria[87,88] and the effect of HDACi on the mitochondrial transmembrane potential ($\Delta\Psi_m$)[37,38,61]. Analysis of the acetylome revealed for the first time that this mechanism of regulation is as complex as the known pathways of protein phosphorylation, although no clear signaling cascades have be established so far[10,82].

Besides the already discussed effects *via* acetylation of transcription factors like STAT1, STAT3, Nf-κB and others[10], HAT and HDAC enzymes are also part of multi-enzyme complexes involved in post-transcriptional pre-mRNA processing, mRNA stability and translation, e.g. by interfering with DNA methyltransferases[89,90]. Recently, an influence of HDACi on miRNA expression profiles has been described[91].

Protein acetylation contributes to the protein turnover *via* proteasomal and non-proteasomal pathways[92]. Interestingly, acetylation can either prevent ubiquitination and subsequent proteasomal degradation (e.g. for p53 or p73) or enhance the degradation of tagged proteins (e.g. HIF-1α or pRb)[93,94]. The stabilization of proteins by acetylation has been shown to involve sites also necessary for the effect of ubiquitin ligases, e.g. at C-terminal lysine residues of p53[95]. In contrast, HDACi have also been shown to induce the expression of E2 and E3 ubiquitin conjugases and ligases Ubc8 and RLIM that can then lead to enhanced ubiquitination of target proteins[96,97]. Although these contradicting phenomena are not completely solved, most reports show a synergistic effect of HDACi with a proteasome inhibitor[98-101]. The tolerability of these combination therapies still needs to be investigated in clinical trials.

DACI AND THE UNFOLDED PROTEIN RESPONSE

The correct folding of proteins by chaperones is a crucial step for homeostasis and maintenance of cellular physiology. Chaperones like heat shock proteins (Hsp) are also a direct target of deacetylases[102]. Hyperacetylation inactivates chaperone function, e.g. of Hsp90, which then leads to misfolding and degradation of Hsp90 target genes[103,104]. This pathway has been demonstrated, among many others, for the oncogene bcr-abl[105] and the receptor tyrosine kinase Erb2[67].

Chaperone function also plays a critical role in the activation of the unfolded protein response (UPR). This pathway is part of a cellular stress response when physiologic conditions of protein folding at the endoplasmic reticulum (ER) are disturbed[106], e.g. after nutrient deprivation or by hyperacetylation of lysine residues[107]. ER stress and UPR can lead to apoptotic cell death by activation of caspase 4/12[108-110] and inhibition of p53[111]. Vorinostat was recently shown to inhibit the function of the chaperone protein GRP78 which leads to activation of PERK and execution of apoptosis[112]. Interestingly, our own data with the pan-deacetylase inhibitor panobinostat (LBH589) also shows that apoptotic cell death is not achieved *via* the canonical intrinsic or extrinsic pathways but by the contribution of the UPR with activation of caspase 12[39]. However, it is unclear if this mechanism is activated *via* hyperacetylation of Hsp chaperones or by so far unknown proteins that might e.g. regulate protein export from the ER or the Golgi apparatus. ER stress and the UPR can also activate autophagy as another means of clearing misfolded proteins, which can also lead to cellular death path-

ways[113]. Several HDACi have been shown to induce autophagic cell death[114-117] and interestingly, vorinostat could also abrogate the survival mechanism of autophagy[118].

CONCLUSION

Deacetylase inhibitors interfere with a plethora of cellular proteins that are involved in regulating central processes of cellular homeostasis and survival. In cancer cells, the interference with these pathways opens new ways for therapy and the current pharmacologic inhibitors of deacetylases are clearly potent agents with more effects than only chromatin remodeling. Further studies are thus urgently warranted to depict the above described pathways and mechanisms more clearly to gain a better understanding of the high tumor cell specificity of these compounds.

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