Histone deacetylase inhibitors as cancer therapeutics

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Abstract: Cancer cells contain significant alterations in their epigenomic landscape, which several enzyme families reversibly contribute to. One class of epigenetic modifying enzymes is that of histone deacetylases (HDAC), which are receiving considerable scrutiny clinically as a therapeutic target in many cancers. The underlying rationale is that inhibiting HDACs will reverse dysregulated target gene expression by modulating functional histone (or other) acetylation marks. This perspective will discuss a recent paper by Markozashvili and co-workers which appeared in Gene, which indicates that the mechanisms by which HDAC inhibitors (HDACis) alter the epigenetic landscape include widespread alternative effects beyond simply controlling regional epigenetic marks. HDACs are involved in many processes/diseases, and it is not surprising that HDACis have considerable off-target effects, and thus a major effort is being directed toward identification of inhibitors which are selective for HDAC isoforms often uniquely implicated in various cancers. This Perspective will also discuss some representative work with inhibitors targeting individual HDAC classes or isoforms. At present, it is not really clear that isoform-specific HDACis will avoid non-selective effects on other unrecognized activities of HDACs.

Keywords: Histone deacetylase (HDAC); histone deacetylase isoforms; histone deacetylase inhibitors; cancer; epigenetics

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It is now generally recognized that cancer cell genomes exhibit global changes in their epigenetic landscape compared with non-transformed cells. There are several types of enzymes known to be involved in epigenetic modifications, including DNA and histone methylases and demethylases as well as histone acetylases and deacetylases. They produce reversible enzyme-mediated markers, and thus they have emerged as potential therapeutic targets for many cancers. Of particular interest clinically are the histone deacetylases (HDACs), which are part of a protein HDAC superfamily (EC 3.5.1.98) (1). This class of enzymes removes acetyl groups from an ε-N-acetyl lysine amino acid on histones (an activity opposite of that of histone acetyltransferases, which add the acetyl groups). Over the years, it has become clear that the H in HDAC is a misnomer, since their targets include many non-histone proteins (2,3). Most HDACs (Class I, II, and IV) are "classical" HDACs, in that they have a zinc-dependent active site, whereas Class III HDACs are referred to as sirtuins (SIRTs) and have a different mechanism (NAD^+-dependent) of action. Class I HDACs (named HDAC1, 2, 3, and 8) are primarily nuclear, although HDAC3 is plasma membrane-associated and is found in both nucleus and cytoplasm (4). Class II HDACs (named HDAC4-7, 9, and 10) are found in nucleus and cytoplasm (5), and HDAC6 is unusual in that it is cytoplasmic and microtubule-associated.

In a recent study which is the focus of this perspective, Markozashvili *et al*. (6) examined the effects of HDAC inhibitors (HDACis) in mantle cell lymphoma (MCL), a relatively uncommon form of non-Hodgkin lymphoma, asking the question as to whether the effects of HDACis were the result of the expected epigenetic modifications directly on the characteristic translocated loci. The unexpected short answer was no.

MCL is directly linked to the t(11:14) translocation, with overexpression of the CCND1 gene, coupled with a

dysregulation of the cell cycle (generally with inactivation of p53 or ATM) (7). Previous work had shown that the transcriptional up-regulation is not explainable simply by translocation of IGH enhancers into proximity of the CCND1 gene, but rather that the t(11:14) translocation also led to a relocalization of the CCND1 locus to a perinucleolar region where it was regulated by nucleolin (8). Chromosomes within nuclei are arranged in specific "chromosomal territories" (9), and chromosomal translocations are known to be accompanied by global relocalization of genes (8,10). Their previous work had demonstrated that gene overexpression in MCL after the t(11:14) translocation has a epigenetic background, and here they investigated whether HDACi effects were the result of its epigenetic action directly on the translocated loci. What they found instead was that HDACis affect the transcription and epigenetic signature of only a small subset of genes, while inducing global changes in nuclear chromosomal architecture.

Using H3K9me3 as a constitutive heterochromatin marker, large heterochromatin clusters were observed in non-treated cells, whereas these clusters rapidly disintegrated with HDACi treatment (6). By 24 h, the global level of H3K9me3 dramatically decreased in all cell lines (both control and MCL), showing that HDACi induced global constitutive heterochromatin disaggregation in both normal and cancer cell lines.

H3K9 acetylation (a prominent marker for transcriptionally active genes) did not simultaneously increase everywhere in the genome as dogma would predict. Indeed, only a small subset of genes reacted to HDACi treatment. Only those genes which showed upregulation with the t(11:14) translocation showed significant changes in their H3K9 acetylation (and dimethylation as HeK9me3, a prominent marker for transcriptionally silent chromatin) status. In general, gene promoters were protected from expected global histone hyperacetylation induced by HDACis, suggesting that the global changes in histone modification levels may alternatively result from a non-epigenetic mechanism(s) of action. A similar effect was also noted previously by Halsall *et al.* (11).

Many non-selective HDACis are currently being explored clinically, either alone or in combination with chemotherapeutics or other agents such as proteasome inhibitors. Predominant responses to HDACi generally include inhibition of proliferation and induction of cell death, which is linked to efficacy in experimental models, and tumor cell "intrinsic" responses may include modulating tumor immunogenicity (12), priming the immune response by increasing expression of tumor-associated antigens and immune-regulated genes, as well as modulating chemokines and cytokines involved in immune system activation (13,14). It has also become clear that effects of HDACis are highly dependent upon context and not easily anticipated. For example, HDACis have been shown to stimulate de-differentiation of human triple-negative breast cancer cells (15), and the HDACi-induced cancer stem cells exhibited a distinct (high pentose pathway activity) metabolic state (16).

Some HDACis have been approved clinically for treatment of various cancers, including leukemia and lymphomas, where they have shown very promising results. HDACis in combination with proteasome inhibitors have also produced excellent results in multiple myeloma (17). Unfortunately, in the clinical setting HDACis often produce serious side effects which limits their utility. Since altered HDAC expression has been implicated in many divergent processes/diseases (including inflammation, calcium homeostasis, cardiovascular diseases, chronic pain, cancer-associated angiogenesis, myogenesis, memory, bone and skeletal disorders), it is not surprising that myriad untoward side effects could arise from their modulation. This has led to the pursuit of isoform- and/or class-selective HDACs to enhance tolerability (17). The question which arises is will this strategy circumvent non-specific effects?

There are a number of recent studies which describe the functional importance of specific HDAC isoforms in divergent settings, with a particular focus on HDAC1 & 2, HDAC6, and HDAC8.

Experimentally, selective inhibition of Class I HDACs1 & 2 was shown to result in cell death with urothelial carcinoma cells (18), using both siRNA or class-specific HDACis (with siRNA knockdown, HDAC1 & 2 had to be targeted concurrently because single knockdowns were accompanied by compensatory up-regulation of the other isoform). However, while use of siRNA and Class I-specific HDACis both reduced proliferation and induced cell death, the mechanisms by which they achieved their ends differed substantially. siRNA knockdown induced apoptotic cell death, whereas in contrast the selective HDACi produced S-phases disturbances and non-apoptotic cell death. This is a key point, and would seem to clearly indicate participation of other mechanisms even with "Class I-specific" HDACis: these other unidentified mechanisms may reflect other functional properties common to the various Class I (and II) HDAC isoforms, or perhaps alternative targets of HDACis. Inhibition of HDACs1 & 2 has also been shown to target

RAD51 and impair homologous DNA repair, thereby sensitizing AML cells to DNA-damaging agents (19). In colorectal cancer, the synergy between HDACis and DNA-damaging agents appears to be specific for HDAC2 (20).

These experimental findings seem to translate to the clinical realm. HDAC1 & 2 has been shown to independently predict mortality in hepatocellular carcinoma (21); increased cancer mortality was significantly associated with HDAC2 expression, and mortality was also increased with high HDAC1 expression.

HDAC6 inhibitors are also of particular interest, since HDAC6 plays a pivotal role in removal of misfolded proteins. As noted, HDAC6 is unusual in that it is cytoplasmic and associated with microtubules, and it deacetylates tubulin and Hsp90 as well as a number of other targets (22), with subsequent downstream effects on various client proteins.

Isoform-specific HDAC6 inhibitors are being tested in combination with proteasome inhibitors for treatment of lymphoid malignancies, where HDAC6-dependent protein disposal currently limits the cytotoxic effects of proteasome inhibitors (23). However, HDAC6 function has also been implicated in a number of cellular processes, and is known to produce deacetylation-independent effects.

Good success has been achieved in development of relatively selective isoform-specific HDACis which have progressed into clinical trials (23). SAHA (a Class I HDAC/HDAC6 co-inhibitor and an autophagy inducer) is being studied in clinical investigations for breast cancer. Targeting HDAC6 (as well as HDAC3), but no other HDAC isoforms (using both siRNA and HDACis) resulted in decreased cell viability, in part via effects on surviving as well as autophagy (24), although it's not clear that the same mechanisms were triggered by the different reagents.

HDAC8-selective inhibitors are also being explored. The p53 pathway is often inactivated in de novo myeloid leukemia through mechanisms which converge on aberrant p53 protein deacetylation, raising the possibility of functionally restoring p53 activity (25). Qi *et al*. (26) specifically looked at AML, which is known to be driven and sustained by leukemia stem cells (LSCs). Although mutation of TP53 is relatively rare in de novo AML, p53 activity is inhibited in AML LSCs via interactions with HDAC8, which aberrantly deacetylates p53 and promotes LSC transformation and maintenance. HDAC8-selective inhibitors were shown to effectively restore p53 acetylation and activity, which subsequently induced apoptosis in AML CD34+ LSCs, while sparing normal cells. This provides some more confidence that isoform-specific inhibitors may have value, although given potentially common non-epigenetic effects, this is not completely clear yet.

Sorting out specific effects for various HDAC isoforms is further complicated by the widespread expression of the multiple HDACs in various leukemia. Yang *et al*. (27) assessed expression levels of all Class I and II HDAC isoforms (HDAC1-10). HDAC expression was generally increased in cell lines and leukemia patients (including AML, CLL and MDS patients), but the patterns of expression were heterogeneous, which could indicate that the role of HDACs in leukemia may be related to global expression (or protein function) rather than specific isoform patterns per se. Various individual isoforms were often transiently increased following HDACi treatment. Van Damme *et al*. (28) also found that HDAC isoform expression was deregulated in CLL B-cells, and that it had a prognostic (albeit complex) clinical significance. Their stepwise regression analysis indicated that HDAC6, 7, and 10 (as well as SIRT3) were independent predictors of treatment-free survival. Poor prognosis was also associated with an overexpression of HDAC7 and 10, but under-expression of HDAC6 (and SIRT3).

As Newbold *et al*. (29) have noted with regard to HDACis, "the field has failed to fully reconcile the biological consequences of exposure to HDACis with the molecular events that underpin these responses". To this I would add that isoform-specific inhibition may also affect unknown molecular functions of other HDACs isoforms unwittingly, which are not related to the Zn-dependent active site, and there are indications that this may be the case from studies comparing HDAC siRNA knockdowns with selective HDACis (18). Thus, "isoform-specific" inhibition may be specific for the particular enzymatic activity attributed to the isoform, but may well have non-isoform-specific effects on different (and perhaps unknown) functions of the other HDAC isoforms.

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Footnote

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