Histone deacetylase inhibitors in cancer therapy: Is transcription the primary target?

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Introduction

Characterization of transcriptional repressor fusion proteins in acute leukemia and growing evidence of the importance of epigenetic changes in cancer onset and progression have stimulated interest in the manipulation of transcription as a mode of cancer therapy. Altering gene expression through chromatin modification now seems to be a viable clinical target. Consistent with this, histone deacetylase inhibitors (HDI) are now being tested for the treatment of leukemia and solid tumors. While these agents show promise, their mechanism(s) of action and selective toxicity against tumor cells have not yet been adequately defined. While the altered expression of specific genes by HDI is important for certain biological outcomes, their effects on other histone-regulated cellular processes, such as mitosis, may be equally important for the antitumor activity.

Aberrant transcriptional repression is common in cancer

Studies from the 1970s indicated that active chromatin was hyperacetylated while inactive, silenced genes were deacetylated and often cytosine methylated. Transcriptional activators can bind and recruit histone acetyl transferases (HAT) while transcriptional repressors and co-repressors interact with histone deacetylases (HDAC) (Melnick and Licht, 2002). In general, binding of repressors and HDACs at promoters correlate with loss of histone acetylation while activator proteins are associated with acetylated chromatin. Furthermore, a more complex code of histone tail modifications including acetylation, methylation, phosphorylation, and ubiquitylation, all potentially amenable to pharmacological modification, has been described to help explain the epigenetic regulation of gene expression (Jenuwein and Allis, 2001). In addition to targeting histone tails, HATs and HDACs can modify other proteins including transcription factors, resulting in altered DNA binding and transcriptional activity.

Aberrant regulation of gene expression is a hallmark of many forms of cancer. In leukemia, chimeric fusion proteins produced by chromosomal translocations alter normal gene regulation through modification of chromatin. The PML-RARα,protein of acute promyelocytic leukemia (APL) and AML1-ETO generated by t(8;21) aberrantly recruits histone deacetylases to target genes leading to repression of otherwise active genes. This results in a differentiation block, contributing to tumor development. Aberrant transcriptional repression may occur following overexpression of nonmutated repressor proteins as in the case of Bcl6 in lymphoma. Altered repression in cancer may also result from overexpression of histone modifying proteins. Histone methylation at particular sites by SET domain proteins is associated with gene silencing (Jenuwein and Allis, 2001). The EZH2 SET protein overexpressed in metastatic prostate cancer might inhibit gene expression in such a manner.

Specific regions of the genome of the cancer cell are frequently aberrantly hypermethylated leading to silencing of tumor suppressor genes such as *INK4A* (Jones and Baylin, 2002). Methylated cytosine residues bind factors like MECP2 that can further recruit DNA methyltransferase and HDAC-containing complexes to propagate and/or maintain gene silencing. How genes become selectively hypermethylated is for the most part unknown; however, the process may be initiated by oncogenic transcription factors such as PML-RARα, which can recruit DNA methyl transferases, leading to silencing of RARα targets (Di Croce et al., 2002). DNA methylation is a dominant silencing process and in general methylated genes cannot be reactivated by histone hyperacetylation alone. Reexpression of methylated genes may in some cases be achieved by treatment with 5-Azacytidine (5Aza), which sequesters DNA methyltransferases. However, the combination of 5Aza and an HDI can synergistically reactivate silenced genes (Jones and Baylin, 2002).

The reversal of aberrant gene repression could thus benefit a wide range of malignancies. Furthermore any component of the repression complex; DNA, transcription factor, co-factor, or modifying enzyme might be a therapeutic target. While protein-protein and protein-DNA interactions are considered very difficult to inhibit pharmacologically, enzymes like the HDACs are considerably more tractable. Accordingly, a number of HDI are now in preclinical development and early clinical trial.

Histone deacetylases and their inhibitors

HDACs are divided into three classes. Class I HDACs are generally nuclear and associate with transcriptional repressors and co-factors. Class II HDACs are larger proteins and can shuttle between the cytoplasm and nucleus. HDAC6 contains two catalytic domains, one of which is specific for tubulin and not for histones (Hubbert et al., 2002). This highlights the important point that HDACs are protein deacetylases and many of the effects of these proteins and their inhibitors could be mediated by modification of nonhistone proteins. Class I and II HDACs are similar to a bacterial histone deacetylase-like protein (HDLP) with a catalytic pocket at the base containing a critical zinc ion and a hydrophobic pocket allowing for the insertion of a lysine side chain. Class III HDACs or sirtuins require nicotinamine adenine dinucleotide as a 1:1 co-factor with substrate. These enzymes may sense the metabolic state and age of the cell (Grozinger and Schreiber, 2002). Mammalian SirT1 deacetylates p53 altering its function as a tumor suppressor (Langley et al., 2002) while SirT2 is a microtubule deacetylase (North et al., 2003).

Both naturally occurring and synthetic HDI have been characterized (Johnstone, 2002; Kelly et al., 2002). HDI are structurally diverse, ranging from simple compounds (i.e., butyrate) to more complex agents such as hydroxamic acids (i.e.,

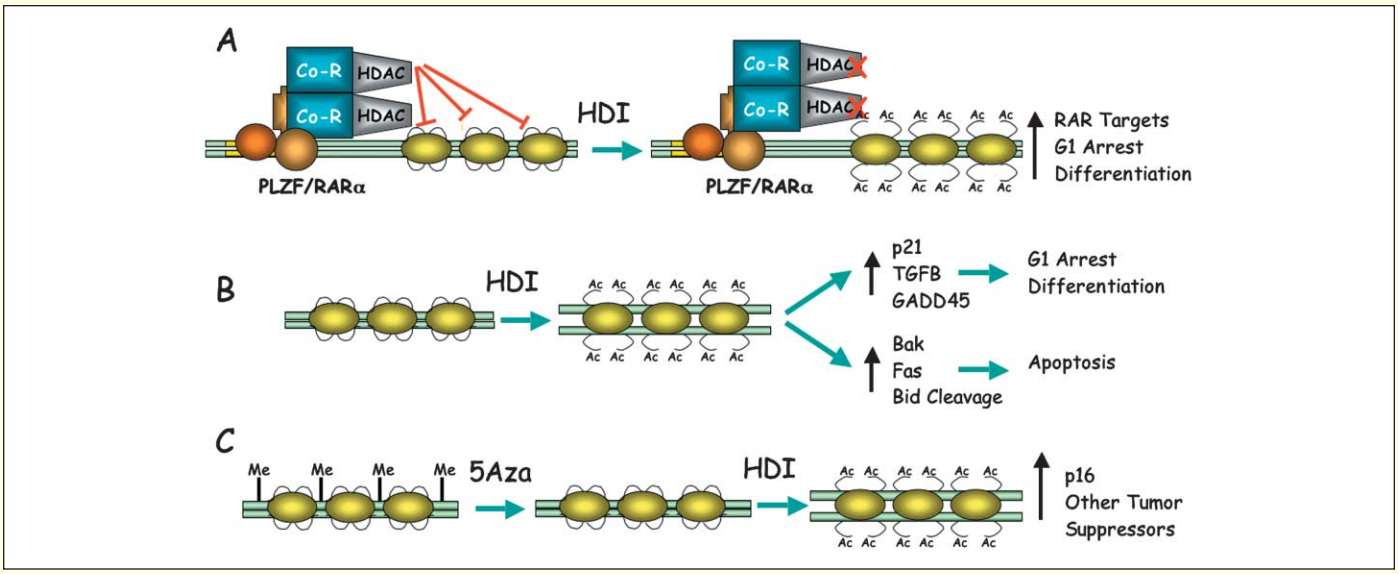

Figure 1. Transcriptional models of the anticancer actions of HDIs

A: HDIs Reverse the repressive effects of fusion transcription factors on target genes.
B: HDIs induce $p21^{WAF1/CIP1}$ to cause G1 arrest and differentiation. Proapoptotic genes may be induced as well.
C: HDIs in combination with 5-Aza reactivate silenced tumor suppressor genes.

suberoylanilide hydroxamic acid [SAHA]), cyclic teratpeptides (i.e., depsipeptide), and benzamides (i.e MS-275). Hydroxamic acids are potent HDI and contain a functional group that interacts with the critical HDAC zinc atom, a 5-6 carbon aliphatic chain which mimics a lysine side chain, and a hydrophobic cap moiety which interacts with the edge of the catalytic pocket and could play a role in HDAC selectivity (Grozinger and Schreiber, 2002). In contrast, HDI with lower potency such as phenylbutyrate and valproic acid (VPA) possess an acyl group to contact the catalytic zinc ion but cannot make significant contact with the catalytic pocket due to their very short side chains. In general, HDI show broad activity with most HDIs able to inhibit the activity of the more than ten Class I/II HDACs. Exceptions include the hybrid cyclic tetrapeptide/hydroxamic acid peptides (CHAP) which have a preference for HDAC1 and 4 over HDAC6, and the recently identified tubacin which specifically targets the tubulin-specific catalytic domain of HDAC6 and does not affect histone acetylation (Haggarty et al., 2003). A specific HDI might be desired if a particular HDAC forms part of an oncogenic repressive complex. However, given the large number of HDACs present in most cells and the prospect of functional redundancy, it is not clear if isoform-specific HDI will be advantageous for most clinical purposes.

HDIs can have a range of antitumor activities including induction of cell cycle arrest, stimulation of differentiation, and provocation of apoptosis. The simplest, almost dogmatic explanation of these effects is that HDIs primarily target transcription to induce their biological effects. This idea has not yet been rigorously proved.

HDIs induce differentiation in acute leukemia

In situations where altered gene expression is clearly linked to disease onset and progression, relief of transcriptional repression may have a beneficial clinical effect. HDIs relieve repression of reporter genes mediated by chimeric oncoproteins such as PML-RARα, PLZF-RARα, and AML-ETO and induce differentiation and terminal division of cells harboring these translocations when combined with all-trans retinoic acid (ATRA) or granulocyte colony stimulating factor (G-CSF). APL associated with PLZF-RAR is insensitive to ATRA therapy due to the constitutive ability of PLZF to recruit HDACs. However, treatment of these leukemic cells with ATRA + trichostatin A (TSA) or SAHA results in cell differentiation and significant apoptosis (He et al., 2001) (Figure 1A).

Can modulation of transcription to force the differentiation of the tumor be extended to other forms of leukemia and cancer? Pediatric tumor cells harboring the EWS-FLI fusion protein exhibit decreased expression of TGFβ-receptor II; this can be reversed by treatment with MS-275 consistent with a chromatin/transcriptional effect (Jaboin et al., 2002). Moreover, leukemic blasts from patients without the characteristic APL chromosomal translocation differentiated with a combination of ATRA and TSA or VPA (Ferrara et al., 2001; Gottlicher et al., 2001). ATRA alone did not induce differentiation, but the combination stimulated known RARα target genes. Unfortunately, the chromatin state of these targets was not examined, and hence formal proof that the HDIs affected chromatin to stimulate differentiation is lacking. Nevertheless, it appears that HDIs release a block to RARα function in many forms of AML. This could be due to modulation of chromatin at retinoic acid receptor (RAR) target loci or even due to modifications of the RAR itself. The notion that HDIs may extend the therapeutic effects of ATRA into other forms of leukemia led our group to initiate clinical trials of VPA in leukemia.

HDI monotherapy induces cell cycle arrest, cell maturation, and changes in gene expression

Treatment of tumor cell lines with HDIs often induces G1 cell cycle arrest and differentiation (Figure 1B). These effects are correlated with the transcriptional activation of *CDKN1A*,

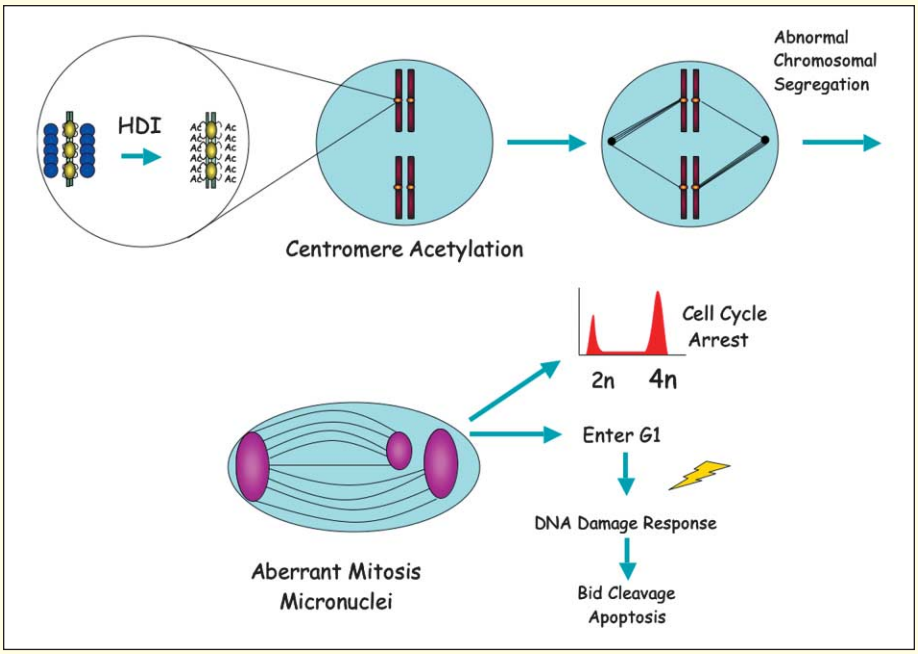

Figure 2. Mitotic model of anticancer effects of HDIs

Hyperacetylation of the centromere induces heterochromatin protein release, centrosome dysfunction, and aberrant mitosis. Cells can arrest in G2 or enter G1 and attempt to replicate damaged DNA, which activates an apoptotic cascade.

encoding the CDK inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner (Richon et al., 2000). Induction of $p21^{WAF1/CIP1}$appears to be important for HDI-induced differentiation as it can initiate myeloid differentiation and cells devoid of $p21^{WAF1/CIP1}$ are resistant to the differentiation effects of HDIs (Archer et al., 1998). Cell cycle arrest by HDIs may also be mediated by altered expression of cyclins A and D and $p27^{KIP1}$, resulting in decreased activity of cdk4 and cdk2 (Johnstone, 2002). Other potential growth inhibitory mechanisms include induction of other cell cycle regulatory genes such as GADD45α and β (Chen et al., 2002) and upregulation of the TGFβ receptor signaling, leading to repression of c-*myc* and cell cycle arrest (Jaboin et al., 2002). In these situations, the growth arrest mediated by HDI is proposed to occur through the direct effects of HDI on chromatin architecture at specific loci leading to changes in gene expression.

Gene profiling studies indicate that HDI modulate only 4%–12% of genes and surprisingly, a similar proportion of genes are activated and repressed, although often with different kinetics. Many of the same genes are regulated by structurally diverse HDIs, suggesting that certain loci are in a highly plastic state and that the HDIs can converge on common targets. In colon cancer cells, TSA and butyrate regulated a similar set of genes with different kinetics, correlating with the more rapid induction of histone acetylation by TSA (Mariadason et al., 2000). SAHA and TSA induced a similar set of genes in breast cancer cells while MS-275, which had no effect on tubulin acetylation, regulated a different set of genes, underscoring the possible importance of nonhistone targets of HDIs (Glaser et al., 2003). The reason for the relatively low and select number of genes affected by a global change in histone acetylation probably stems from the hierarchical nature of epigenetic transcriptional control where DNA and histone methylation can provide a dominant-repressive effect over histone acetylation. Consistent with this, treatment of colon cancer cells with HDI or 5Aza alone induced a different set of genes while combination therapy synergistically activated a subset of methylated genes, including a number of putative tumor suppressors (Yamashita et al., 2002) (Figure 1C). Although altered gene regulation certainly occurs in tumors treated with HDIs, other than *CDKN1A*, it is uncertain how essential these genes are for the reprogramming of the cancer cell to differentiate or cease growth.

The alternative mechanism-HDIs induce apoptosis

HDIs can induce apoptosis of the cancer cell, and their ability to activate proapoptotic genes such as Fas and Bak suggests that transcriptional regulation by HDIs may play some role in the process (Johnstone et al., 2002) (Figure 1B). However, in several systems, it appears that transit of cells through the G1/S boundary and the accumulation of cells with a 4n DNA content are precursors to cell death. As with many anticancer drugs, the intrinsic apoptotic pathway involving activation of proapoptotic BH3-only Bcl-2 proteins and mitochondrial membrane disruption is central to HDI-mediated cell death (Figure 2). For example, SAHA-induced apoptosis is associated with activation of the BH3-only proapoptotic protein Bid, generation of reactive oxygen species, and release of cytochrome c. Apoptosis induced by SAHA and other HDIs is only partially blocked by a polycapsase inhibitor, but almost completely blocked by overexpression of Bcl2 (Ruefli et al., 2001; Henderson et al., 2003), emphasizing the importance of the intrinsic pathway but suggesting that noncaspase-mediated events downstream of the mitochondria are at work. SAHA-mediated Bid cleavage and apoptosis was blocked by a calpain inhibitor (Mitsiades et al., 2003) and was cycloheximide dependent (Ruefli et al., 2001), but whether SAHA directly modulates gene expression to activate the calpain/mitochondrial apoptosis pathway is unknown.

The importance of p53 in HDI-mediated cell death is uncertain. p53 is deacetylated and destabilized by class I and III HDACs. Hence HDIs could augment p53 function to activate genes encoding BH3-only proteins (i.e., PUMA, Noxa) and thereby initiate the intrinsic apoptotic pathway. While most studies have indicated that HDI-mediated apoptosis can occur in the absence of wild-type p53,a recent study using dominant-negative p53 suggested that HDI-mediated apoptosis required p53 (Henderson et al., 2003). A number of other apoptotic mechanisms were described for HDIs including stimulation of JNK activity and induction of Fas, Fas ligand and sensitization of cells to the extrinsic pathway (Aron et al., 2003). These reports are difficult to interpret, due to the fact that each study typically utilizes different cell lines and different HDIs. A more systematic study of several HDIs in the same cell line is needed, preferably in cells with known mutations in genes critical for the regulation of cell cycle arrest or apoptosis. The HCT116 cell line and sub-

Table 1. How histone deacetylase inhibitors may function in cancer therapy
Inhibition of cell proliferation
Augmentation of nuclear receptor response driving terminal cell differentiation
Reversal of repression by fusion transcription factors or overexpressed repressors
Induction of p21, G1 arrest, and cellular differentiation
Reactivation of silenced tumor suppressor genes in combination with DNA methyl transferase inhibitors
Suppression of telomerase gene expression
Induction of apoptosis
Activation of calpain/mitochondria-dependent apoptosis
Activation and or sensitization of death-receptor mediated killing
Mitotic dysfunction, aberrant chromosomal segregation, and DNA damage
Induction of topoisomerase II may alter sensitivity to DNA-damaging agents
Other mechanisms
Alteration of angiogenic signaling
Alteration of microtubule function
Induction of MHC antigens on the cell surface to augment immune responses
Suppression of IL-2-mediated gene expression

clones in which p53, p21, and Bax have been deleted would be a good starting point. Such experiments might also determine which effects of structurally diverse HDIs are shared and attributable to inhibition of HDACs and which may be more specific to the ability of the agents to affect other pathways.

HDI action may be unrelated to direct effects on gene expression

While it is clear that HDIs can directly modulate gene expression through histone hyperacetylation, this may not be the critical determinant of their antineoplastic actions. Histone-mediated changes in gene expression per se may not be responsible for apoptosis induced by HDIs, yet histones may still be their primary targets. Histone acetylation in heterochromatin is tightly regulated during S phase, and disruption of this process triggers cell cycle arrest within G2/M. The HDI-associated G2 checkpoint may be related to HDI-mediated hyperacetylation of the centromere, allowing release of heterochromatin proteins resulting in abnormal chromosomal segregation and nuclear fragmentation (Taddei et al., 2001). In cancer cells, the HDI-associated G2 checkpoint can be lost, leading to aberrant mitoses and apoptosis (Qiu et al., 2000). Hence, it is formally possible that dysregulated histone acetylation during S phase and/or mitosis, rather than modulation of gene expression, may be the major event necessary for induction of HDI-mediated cell death (Figure 2).

Why are cancer cells sensitive to HDIs?

The basis for the selective toxicity of cancer cells to HDIs is unclear. If altered gene expression were the primary mechanism for HDI-induced apoptosis, then one would expect both normal cells and tumor cells to be equally sensitive. In fact, given that the disruption of apoptotic pathways is an essential event in tumorigenesis (Johnstone et al., 2002), it might be expected that normal cells would be more sensitive than tumor cells to HDI-induced death. If however HDI-induced apoptosis is related to aberrant mitosis, then the fact that cancer cells universally lose cell cycle checkpoints can make these cells inherently more sensitive to the agents. In support of this, leukemia cell lines treated with HDI initially accumulate with a 4n DNA content and then undergo apoptosis. However, if these cells are manipulated to undergo arrest in G1 through overexpression of $p16^{INK4a}$, Bid is no longer activated and the cells remain viable, although global histone hyperacetylation still occurs (R.W.J., unpublished data). Hence the loss of the G1 checkpoint, by loss of $p16^{INK4a}$ or another mode of subversion of RB function, a virtually universal occurrence in tumors, might explain the increased sensitivity of cancer cells to HDIs.

A further unsolved question is what factors determine whether a cancer cell undergoes cell cycle arrest, differentiation, or death in response to HDIs. Drug levels are one factor as most HDIs exhibit toxicity at higher doses and induce G1 arrest at lower doses. Whether this is exclusively due to the effects of HDI on chromatin remodeling and gene transcription is unknown. Tissue type is another factor. At the same dose of drug, one type of cell may undergo G1 arrest and differentiation while another promptly undergoes apoptosis. Why this occurs is completely obscure but might be related to the intracellular metabolism of the drug or the specific subset of genetic defects affecting regulation of the cell cycle or apoptosis in a particular cancer cell. In support of the latter idea, the preexisting apoptotic set point of the cell can alter the balance between HDI-induced growth arrest and cell death. For example, cells engineered to overexpress Bcl2 are resistant to HDI-mediated apoptosis but the effects on the cell cycle are unaltered (Johnstone, 2002). Furthermore, as noted, the decision of a cell to arrest in G1 or progress through the cell cycle and experience the effects of HDIs on mitosis can determine cell fate. In support of this idea, there is an inverse correlation between $p21^{WAF1/CIP1}$ induction and cell cycle arrest and apoptosis (Burgess et al., 2001). If cells treated with HDI either cannot induce $p21^{WAF1/CIP1}$due to overriding epigenetic changes at the CDKN1 locus or have functional defects in the G1 checkpoint, then the cells may be destined to undergo apoptosis. We propose that the possible interplay between direct effects of HDI on transcription and the indirect effects triggered by aberrant mitosis determine the ultimate biological outcome.

HDIs in clinical trial

HDIs as single agents have entered clinical trials (Kelly et al., 2002). Phenyl butyrate was administered orally to treat solid tumors and by continuous infusion in the myelodysplastic syndrome (MDS). A few patients in each trial had evidence of stable disease or mild improvement. In these studies, it was difficult to maintain drug levels and pulse dosing might be considered as an alternative. A trial of depsipeptide achieved therapeutic levels and one patient with cutaneous T cell lymphoma achieved a complete response. Clinical trials of MS-275 (National Cancer Institute), SAHA (Memorial Sloan Kettering Cancer Center), Valproic acid (Mount Sinai School of Medicine), and others are underway. In these studies, it will be critical to correlate clinical response, accumulation of acetylated chromatin, changes in gene regulation, and apoptosis. This is especially important given the multiple ways by which HDIs may inhibit cancer cell growth and survival (Table 1).

The transcriptional regulation model of HDI activity predicts that combining HDIs with other agents will augment transcrip-

tion of target genes and lead to differentiation. ATRA and HDIs augment differentiation of APL and other types of myeloid leukemia in vitro. In addition, HDIs plus G-CSF or 5 Aza induce differentiation of leukemia cells harboring the AML1-ETO fusion protein. Inhibition of DNA methylation with 5Aza reactivates epigenetically silenced genes and azacytidine induces improvement in hematopoiesis in MDS. In one study, this was associated with upregulation of $p16^{INK4a}$ (Daskalakis et al., 2002). Successor trials are planned to treat MDS with the combination of HDIs and 5Aza. On one hand, this therapy might lead to further upregulation of silenced genes, growth arrest, and differentiation of the malignant cells. However, it is equally likely that DNA damage induced by 5 Aza, along with changes in the apoptotic threshold induced by HDIs, could lead to selective apoptosis of the malignant cells and regrowth of normal clones. Translational experiments that must be included in these trials to prove that differentiated hematopoietic cells derive from a malignant precursor should include fluorescence in situ hybridization for marker chromosome detection, histone acetylation studies, monitoring of differential gene expression, and apoptosis.

Should the counter theory that HDIs reset the apoptotic set point be correct, combination therapy may still be required to elicit the full effect of these agents. Such therapy might include withdrawal of survival signals through RTK blockade, induction of DNA damage by chemotherapy or radiation therapy, or induction of TNF receptor pathways.

Perspective

The study of HDIs in cancer, initially motivated by the study of aberrant transcriptional repression, is rapidly evolving. It is now apparent that the transcriptional effects of HDIs may be but one facet of their action. In specific forms of cancer such as the myeloid leukemias, HDIs may indeed be working as predicted to block histone deacetylases and potentiate acetylation at specific genes. However, the focus on histone acetylation neglects the many other facets of epigenetic regulation of gene expression. True targeting of transcription in leukemia and other tumors might require a combination of agents to modify chromatin, including DNA methyl transferase inhibitors, histone methylase inhibitors, HDIs, and specific stimulators of transcription factor activity.

In the more common forms of cancer, HDIs are also promising therapeutic agents, but here their mechanism of action may be quite different. HDIs have pleiotropic effects on cells, with outputs of differentiation, growth arrest, and cell death all possible. It is not clear if transcription is the primary HDI target in these cells, or if other effects, perhaps triggered by aberrant mitosis, play a fundamental role. In patients, these effects may be dependent on the particular genetic lesions of the tumor, the dose and length of exposure of the tumor to drug, and the possibility that HDIs could affect secondary signaling pathways. A combination of basic, clinical, and translational studies will ultimately determine the clinical utility of these agents and their mechanism of action.

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