Activity of the histone deacetylase inhibitors LBH589 and LAQ824 in hematologic malignancies

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Histone deacetylase (HDAC) inhibitors (HDIs) are potent inducers of *in vitro* growth arrest, differentiation and apoptosis of human leukemia and lymphoma cells as well as exert *in vivo* anti-leukemia and anti-lymphoma effects. The hydroxamic acid (HA) analogue class of HDIs, e.g., LBH589 and LAQ824, have been shown to induce not only the acetylation of the lysine residues of core nucleosomal histones but also of transcription factors and other important proteins. Thus, they should be referred to as protein deacetylases. Together, HA-HDI-induced modifications of proteins mediate the biologic the therapeutic effects, which are currently being investigated through pre-clinical and clinical studies in hematologic malignancies. This review briefly describes the current status of the development of these agents in the therapy of hematologic malignancies.

Histone and other protein acetylation/deacetylation

HATs (histone acetyl transferases) and HDACs are the two classes of enzymes that mediate the acetylation and deacetylation, respectively, at the evolutionarily conserved N-terminal lysine residues of the core histones. Therefore, the acetylation status of the chromatin associated with particular genes is dictated by the balance between the activities of HATs and HDACs. In general, while increased histone acetylation is associated with open and active chromatin and increased transcription, deacetylated histones are associated with condensed chromatin and transcriptional repression. HATs and HDACs are recruited to gene promoters by multiprotein transcriptional complexes, where they regulate transcription through chromatin modification without directly binding the DNA. HDACs exist in large multimeric complexes that are recruited to the gene promoters by co-repressors, e.g., Sin3, N-CoR (nuclear receptor co-repressor), SMRT (silencing mediator for retinoic acid and thyroid hormone receptor), which are associated with transcription factors. Importantly, HATs and HDACs have also been shown to affect the acetylation status of the lysine residues of transcription factors, e.g., p53, E2F1, GATA1, RelA and hormone receptors, which may affect their DNA binding and transcriptional activity. Additionally, HDACs have been shown to deacetylate proteins other than histones or transcription factors. These include the cytoskeleton protein α-tubulin, nuclear import protein importin-α7, signal transduction protein β-catenin, DNA repair enzymes Ku70, and most recently the heat shock protein 90 (hsp90). This creates the possibility that modulation of the function of specific HDACs may not only affect gene transcription but also modify the function of other proteins involved in important biologic functions in the cancer cell. Therefore, HDACs should be best characterized as protein deacetylases.

Aberrant HAT and HDAC activity in leukemia

Abnormal activity of HATs and HDACs resulting in aberrant gene transcription is commonly observed in cancer cells, and is especially a hallmark of leukemia and lymphoma. Chimeric fusion oncoproteins in leukemia have also been shown to undergo inappropriate forced dimerization. This alters the association of the DNA binding portion in the chimeric oncoproteins with its transcriptional cofactors, which recruit HDACs to the promoters and repress genes involved in cell cycle growth inhibition and differentiation. For example, in AML the dimerization domains in the partners of the core binding factor (CBF) AML1 (also known as RUNX1 or CBFA2), including TEL, ETO and MTG16, also mediate transcription repression by recruitment of co-repressor-HDAC complexes. The fusion AML1-ETO protein suppresses AML1 responsive promoters and represses the normal AML1 target genes, leading to block in myeloid differentiation. In chromatin 16 inversion leukemia, another CBF, CBFβ, fuses with smooth muscle myosin heavy chain gene MYH11, which

recruits transcriptional repressors and HDACs to dominantly repress AML1 transactivated genes. In APL variants, PLZF-RARα or STAT5β-RARα, represses RARα mediated transcription by recruiting N-Cor/Sin3/HDAC1 complex, which is not reversed by treatment with ATRA. This results in chromatin modification and repression of genes leading to blocked differentiation and inhibition of apoptosis. Similar to PLZF, the BTB/POZ domain containing transcriptional repressor Bcl-6 can recruit corepressor (N-CoR, BCoR or SMRT) and HDAC, which is required for the survival and proliferation of B NHL cells. Collectively, these mechanistic insights support the rationale to develop inhibitors of HDAC activity and point to their potential therapeutic use in correcting the transcriptional deregulation of genes involved in cell cycle regulation and apoptosis.

Classes of HDAC Inhibitors (HDIs)

Structurally diverse classes of naturally occurring and synthetic compounds have been recognized for their ability to bind to the catalytic pocket of HDACs and chelate the zinc ion at its base, thereby inhibiting pan-HDAC activity. The various HDIs studied so far have been shown to inhibit class I (HDACs 1, 2, 3 and 8) and II (HDACs 4, 5, 6, 9 and 10). Class III HDACs (SIR T1, 2, 3, 4, 5, 6 and 7), also known as sirtuins, require NAD+ rather than zinc as a cofactor for their activity, and are not inhibited by the pan-HDIs that are currently under investigation. Among these are the cinnamic acid hydroxamate HA-HDIs, e.g. LBH589, LAQ824, which are active at the nanomolar concentrations.

HA-HDI-induced growth arrest, differentiation and apoptosis

Treatment with LAQ824 and LBH859 increases the intracellular levels of p21 and p27, induce cell cycle arrest and apoptosis of human AML (cultured: HL-60, MV4-11 and U937, and primary AML) and CML-BC (cultured; K562 and LAMA84, and primary CML-BC) cells, with relative sparing of normal cells. Recent cDNA microarray studies have shown that treatment with HDI modulates the expression of a selective subset of genes consisting of approximately 2-10% of all genes in different cell types, based on the cell type and the HDI used and the design of the experiment, with as many genes upregulated as are downregulated. Since a large number of gene expressions are affected, it has been difficult to assign a mechanistic role to an alteration in a specific gene expression for a specific biologic effect due to HDI treatment. Among the genes consistently upregulated is the CDK inhibitor p21 in a p53-independent manner. HDI-induced expression of p21 is necessary for HDI mediated cell cycle G1 arrest. In $p21^{-/-}$ and $p27^{-/-}$ mouse embryonic fibroblasts, HA-HDI-induced cell cycle G1 arrest but not apoptosis was blocked. Treatment with HDIs has also been noted to repress cyclin D and cyclin A, as well as upregulate p16 (CDKN2 or INK4) and p27, the latter non-transcriptionally. These lead to decreased activity of CDK4 and CDK2 and dephosphorylation of pRb, which also induces cell cycle arrest in G1. Treatment with HDIs has also been shown to transcriptionally downregulate the expression of CTP synthetase and thymidylate synthetase, which are involved in DNA synthesis, thereby inhibiting cell cycle S phase progression.

Treatment with HDIs triggers both the intrinsic, mitochondria-initiated signaling for apoptosis and sensitizes tumor cells to the death ligands that initiate the extrinsic pathway of apoptosis. HA-HDIs such as SAHA, LAQ824 and LBH859 have been shown to upregulate Fas and the Apo-2L/TRAIL receptors DR4 and DR5, down regulate c-FLIP, and enhance Apo-2L/TRAIL-induced DISC (death inducing signaling complex) and apoptosis. HA-HDIs have also been shown to induce mitochondrial permeability transition, which is associated with increased Bak, Bax and Bim levels, but attenuation of Bcl-2, Bcl-xL and XIAP levels. This was shown to release pro-death molecules such as cytochrome c, Smac and Omi into the cytosol, thereby triggering the activity of the Apaf-1 *apoptosome*, which leads to the processing and activation of caspase-9 and -3. Among the HDI-induced upstream events that may trigger the mitochondrial pathway of apoptosis is the upregulation of the multi-domain proapoptotic Bak. In addition, treatment with HA-HDIs has been shown to induce Bax conformation change, which precedes its localization to the mitochondria where it induces the permeability transition. Treatment with HDIs was also demonstrated to induce the cleavage of the proapoptotic, BH3 domain only-containing Bid and generation of reactive oxygen species. While co-treatment with a pan-caspase inhibitor partially blocked HDI-induced apoptosis, it was completely inhibited by Bcl-2 overexpression. In some cell-types, abrogation of HDI-induced p21 augmented HDI-induced apoptosis, suggesting that p21 induction by HDIs dampens the apoptotic effects of HDIs. However, as compared to the p21-expressing colon carcinoma HCT-116 cells, in p21-null HCT-116 cells LAQ824-induced apoptosis was attenuated. As compared to the colon cancer HCT116 cells, LAQ824 also induced significantly less apoptosis of HCT116 cells without Bax, Puma and p53 expression ($p<0.01$); HCT116/Bax/- cells displayed the greatest resistance to apoptosis. Conversely, LAQ824 induced significantly more apoptosis of $HCT116/XIAP^{-/-}$ cells. Although HA-HDI treatment increases the levels of reactive oxygen species (ROS), this occurred after the cytosolic accumulation of pro-death proteins (e.g., cytochrome c, Smac and Omi) and caspase 3 activation.

This makes it unlikely that HA-HDI-mediated ROS induction is mechanistically responsible for HA-HDI-induced apoptosis. Treatment with LBH589 or LAQ824 induces acetylation of α tubulin and heat shock protein (hsp) 90, suggesting that these effects may be mediated by inhibition of HDAC6. Significantly, HDAC6 could be co-immunoprecipitated with hsp90. A 90% attenuation of HDAC6 by RNAi to HDAC6 induced hyperacetylation of hsp90 and α tubulin. Conversely, ectopic overexpression of HDAC6 in K562 cells inhibited HA-HDI-induced α tubulin and hsp90 acetylation. HA-HDI-induced hyperacetylation of hsp90 was associated with inhibition of ATP binding and chaperone function of hsp90, promoting polyubiquitylation and proteasomal degradation of hsp90 client proteins.

A number of oncoproteins in leukemia, e.g., Bcr-Abl and FLT-3, are known to be client proteins for hsp90, and treatment with HA-HDIs lowers the levels of Bcr-Abl (wild type and Gleevec-refractory mutant) and mutant FLT-3, as well as depletes AKT and c-Raf levels in acute leukemia cells. This contributes to the lowering of the threshold for apoptosis in cancer cells. Therefore, treatment with these HDIs, similar to the other well-known hsp90 inhibitors, such as the geldanamycin analogues, induces the polyubiquitylation and proteasomal degradation of Bcr-Abl and mutant FLT-3 in CML and AML cells, respectively. These biologic effects have important implications for designing novel future treatment strategies for human leukemia, where the leukemogenic oncoprotein or the downstream pro-survival proteins are hsp90 client proteins that can be targeted for down modulation by HDI treatment. Taken together, these findings indicate that HA-HDIs induce apoptosis by multiple mechanisms: a. by inducing pro-death and depleting pro-survival proteins; b. by causing hyperacetylation of hsp90 and depleting the levels of those hsp90 client proteins to which leukemia and breast cancers may be addicted for their growth and survival.

HDIs have also been shown to transcriptionally upregulate thioredoxin binding protein 2 (TBP2), while concomitantly reducing the levels of thioredoxin. Taken together, these modulations due to treatment with HDIs could contribute to the lowering of the threshold for apoptosis secondary to antileukemia agents. Recently, in cells in which caspase mediated apoptosis was blocked by pan caspase inhibitors, HDIs were shown to induce caspase-independent non-apoptotic cell death by autophagy, suggesting that if mitochondrial pathway of cell death is blocked HDI-induced autophagic cell death may result.

HA-HDI-induced differentiation effects

HDI-induced growth arrest has also been shown to induce differentiation of leukemia and breast cancer cells. Induction of p21 by HDIs appears to be mechanistically important, since cells lacking p21 are resistant to growth arrest and differentiation induced by HDIs. Treatment with HDI alone or in combination with ATRA or G-CSF has been shown to overcome the inhibition of differentiation due to chimeric fusion oncoproteins such as PML-RARα, PLZF-RARα, or AML-ETO. In addition, a combined treatment with HDI with ATRA induces differentiation of APL cells that are resistant to treatment with ATRA alone. Recently, primary leukemia blasts were shown to undergo differentiation following treatment with a combination with ATRA and HDIs. However, it remains unclear why in some cell-types exposure to HDIs induces growth arrest and differentiation while in other cell-types it results in apoptosis.

HA-HDI-induced antiangiogenic effects.

There is evidence to suggest that treatment with HDIs leads to tumor regression not only by inhibiting cell cycle progression and inducing differentiation and apoptosis, but also through antiangiogenic and immune modulation effects. For example, treatment with HDIs upregulated p53 and VHL and downregulated the hypoxia-inducible factor α (HIF1α) and vascular endothelial growth factor (VEGF), leading to inhibition of angiogenesis *in vitro* and *in vivo*. A variety of other HDIs have also been shown to inhibit VEGF-stimulated endothelial cells and angiogenesis.

Potential basis for the antitumor selectivity of HDIs.

Treatment with HDIs has been shown to induce histone hyperacetylation and upregulation of p21 to a similar extent in normal versus transformed cells. Notwithstanding this, normal cells undergo cell cycle G1 arrest with insensitivity to apoptosis, while tumor cells exhibit cell cycle growth arrest in G1 or G2/M phase followed by differentiation and/or apoptosis. One reason for this differential effect may be that, transformed cells have loss of G1, G2/M and/or mitotic spindle checkpoints, and treatment with HDIs selectively induces aberrant mitosis leading to apoptosis of transformed cells. Transformed but not normal cells may be aberrantly addicted to the deregulated activities of the pro-growth and survival proteins, e.g., Bcr-Abl and mutant FLT-3, as well as of the downstream activity of AKT and Ras/Raf/ERK pathways. Treatment with HA-HDIs may undermine the aberrant transactivation due to oncoproteins on which the hematologic malignancy is clearly dependent for growth and survival, e.g., Bcl-6, AML1-ETO and PLZF-RARα. In leukemia and other transformed cells, treatment with HDI also upregulates the death ligands and their receptors, as well as downregulates the levels and activity of proteins that dampen the death signaling downstream of the death

receptors and death-inducing signaling complex. HDI-induced coordinated and differential modulation of the expression of ROS and thioredoxin in transformed versus normal cells may also mediate the anti-tumor selectivity of HDIs.

Combinations of HDIs with Other Agents

Due to their relative antitumor selectivity and tolerable host toxicity, HA-HDIs have been used in combination with a variety of novel and conventional anticancer agents. These are:

DNA hypomethylating agents

Aberrant methylation is now well known to silence the expression of tumor suppressor and other cancer relevant genes, such as p16INK4A and p14INK4b, Apaf-1 and caspase-8, and the silenced genes can be re-expressed by treatment with DNA methyl transferase (DNMT) inhibitors (MTIs). DNMT and a group of methyl cytosine binding proteins, e.g., MeCP2, can also recruit and direct HDACs to the chromatin associated with silenced genes. DNA methylation is a dominant gene-silencing process, and methylated genes show resistance to re-expression by treatment with HDIs alone. Combined treatment with an MTI and HDI has been shown to be superior in de-repressing silenced TSGs, as well as in inducing increased growth inhibition, differentiation and apoptosis of transformed cells. Best results in de-repressing silenced genes are observed when MTI is used first at relatively low doses followed by exposure to the HDI.

Differentiation-inducing agents

HDIs have also been tested in combination with ATRA against APL cells expressing PML-RARα or PLZF-RARα. Enhanced differentiation and significant apoptosis due to the combination has been reported. The HA-HDI TSA combined with ATRA is especially effective against ATRA-resistant PLZF-RARα containing APL cells. Primary leukemia blasts also demonstrated differentiation following combined treatment with ATRA and TSA or VPA. The combination was noted to induce RARα-targeted genes, suggesting that in AML cells HDIs release a block to RARα-mediated gene expression involved in differentiation.

Cell cycle and cell-signaling modulators

Recent studies have shown that treatment with LAQ824 or LBH589 attenuates the mRNA level of bcr-abl and promotes polyubiquitylation and proteasomal degradation of *Bcr-Abl*. In addition, a combined treatment with LAQ824 and Bcr-Abl tyrosine kinase inhibitor imatinib mesylate or the dual Src/Abl kinase inhibitor was shown to induce apoptosis of imatinib-sensitive or imatinib-refractory CML cells. Recently, LAQ824 or LBH589 were also demonstrated to promote degradation of mutant FLT-3 and induce apoptosis of AML cells containing mutant FLT-3. Additionally, combined treatment with PKC412, a staurosporine-derived-protein kinase inhibitor with a spectrum of activity that includes classic PKCs and FLT-3, and LAQ824 or LBH589 induced synergistic apoptotic effects in AML cells with mutant FLT-3. Taken together, these preclinical findings support the rationale to determine the safety and efficacy of the combinations in which HDIs are administered together with specific inhibitors of oncoprotein tyrosine kinases responsible for pro-growth and pro-survival signaling in transformed cells.

Conventional cytotoxic agents

As discussed above, molecular perturbations due to treatment with HDI lower the threshold for apoptosis by multiple mechanisms. This supports the rationale to combine HDI with conventional chemotherapeutic agents, which generally trigger apoptosis by perturbing cell cycle and/or inducing DNA damage. Consistent with this, co-treatment with LAQ824 was demonstrated to significantly enhance the cytotoxic effects of docetaxel, Epothilone B and Gemzar against human breast cancer cells.

Novel targeted agents

Based on the ability of HDIs such as LAQ824 to lower the threshold for apoptotic signaling initiated both by the extrinsic, death receptor-initiated and intrinsic mitochondrial pathways of apoptosis, the activity of these agents in combination with Apo-2L/TRAIL was pre-clinically tested against human leukemia cells. Co-treatment with SAHA or LAQ824 significantly enhanced APO-2L/TRAIL-induced apoptosis of cultured and primary acute leukemia cells. A similar effect was also seen for SAHA plus APO-2/TRAIL in multiple myeloma cells. Recently, the combined effects of HDIs such as LBH589 with the hsp-90 antagonist, 17-allylaminodemethoxy geldanamycin (17-AAG) were determined in human leukemia cells. The combination was highly synergistic in inducing the mitochondrial pathway of apoptosis. The synergistic effect of the combination was associated with downregulation of Raf-1 and p-AKT, inactivation of MEK and ERK1/2, as well as attenuation of the levels of Bcr-Abl and mutant FLT-3 in leukemia cells. It is noteworthy that combined treatment with LBH589 and 17-AAG showed superior activity than either agent alone in attenuating the levels of leukemia cells expressing imatininb mesylate-refractory Bcr-Abl containing the T315I point mutation that confers high level of clinical resistance against imatinib mesylate, as well as against AMN107 and BMS354825 that have been recently shown to be high-

ly active against imatinib-refractory CML. Bortezomib is a specific and potent inhibitor of the 20S proteasome. Co-administration of minimally toxic concentrations of bortezomib with LAQ824 resulted in marked increase in caspase activation and apoptosis of Bcr-Abl positive leukemia cells sensitive or resistant to imatinib. This was again accompanied with the downregulation of the Raf-1/MEK/ERK1/2 pathway. Taken together, these findings suggest that combined hsp90/histone deacetylase or proteasome/histone deacetylase inhibition may be a promising strategy against leukemia or other hematologic malignancies.

Conclusions

LBH589 and LAQ824 are promising HA-HDIs, which show important pre-clinical activity against human leukemia and lymphoma cells. These agents may benefit more as combination treatment with either conventional or novel targeted agents in the therapy of human hematologic malignancies. Clinical studies of these agents administered alone in hematologic malignancies are currently underway, while combination therapies with other novel targeted agents are planned.

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