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THIOREDOXIN IN CANCER-ROLE OF HISTONE DEACETYLASE INHIBITORS

Paul A. Marks*

Memorial Sloan-Kettering Cancer Center, 1275 York Avenue New York, New York, 10021

Abstract

Increased levels of thioredoxin (Trx) occur in a number of human cancers, which may contribute to the resistance of cancers to therapy by scavenging reactive oxygen species (ROS) which are generated by various anti-cancer agents. Many human cancers have low levels of thioredoxin binding protein (TBP-2). TBP-2 binds to Trx and blocks its reducing activity. Histone deacetylase inhibitors (HDACi) up-regulate TBP-2 in various transformed cells, associated with a decrease in Trx levels. Up-regulation of TBP-2 and decrease of Trx may contribute to the sensitivity of many hematologic and solid tumors to anti-cancer activity of HDACi.

Keywords

Thioredoxin; Thioredoxin Binding Proteins; Histone Deacetylase; Histone Deacetylase Inhibitors; Suberoylanilide Hydroxamic Acid

Introduction

Thioredoxin (Trx) is a 12-kDa thiol reductase which plays an important role in cell proliferation and cancer [1]. Trx system includes Trx reductase, Trx and NADPH. Trx functions in a number of pathways in mammalian cells [1,2]. Trx is a hydrogen donor for ribonucleotide reductase, which is required for DNA synthesis. Trx can act as cellular growth factor, stimulating proliferation of cells, including lymphoid cells, fibroblasts and various transformed cells [3-8]. It has been shown that stable transfection of Trx gene can stimulate cell proliferation [9]. Trx is a potent antioxidant, and act as a scavenger of reactive oxygen species (ROS). Trx reduction of ROS can protect against various forms of cellular stress that can cause cell death [10,11]. Trx has been reported to alter the DNA-binding of transcription factors, including NF- κ B, TF-kC, glucocorticoid receptor and BZLF1 [12-17]. Trx functions as an anti-apoptosis or cell death factor by inhibiting apoptosis signal-regulating kinase-1 (ASK-1), a critical factor in stress induced cell death, as well as, by neutralizing ROS [18].

Thioredoxin in Human Cancers

Trx has been found expressed at relatively high levels in a number of cancers [19]. Trx levels are elevated in certain human lung cancers [20,21], pancreatic cancer, cervical cancer [23] and

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*Corresponding author: Telephone 212-639-6568 Fax 212-639-2861, Paula_Marks.org

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hepatocellular cancer [24]. For example, in pancreatic ductal carcinoma tissue, levels were elevated in 24 of 32 cases as compared to normal pancreatic tissue. Trx expression was associated with aggressive tumor growth and poorer prognosis in primary non-small cell lung carcinomas, and colorectal cancer. In colorectal cancer patients, Trx expression tended to increase with higher Dukes stage and was significantly correlated with reduced survival.

Several lines of evidence suggest that Trx may confer resistance to anti-cancer drugs [25-27]. The resistance of adult T-cell leukemia cell lines to doxorubicin and ovarian cancer cell lines to cisplatin has been associated with increased intracellular Trx-levels. Transfection with an antisense plasmid to Trx restored sensitivity to cisplatin and increased sensitivity to several other cytotoxic drugs. Trx levels were also elevated in cisplatin-resistant gastric and colon cancer cells. Stable transfection of fibrosarcoma cells with Trx resulted in increased cisplatin resistance and high Trx expression is associated with resistance to docetaxel in breast cancer. The cellular level of Trx might be a clinically useful marker for sensitivity to therapy.

Cancer cells are able to adapt to hypoxic conditions found in nearly all solid tumors. Hypoxia leads to activation of hypoxia-inducible factor 1 (HIF-1). HIF binds to hypoxia response elements (HRE) and induces expression of genes that promote angiogenesis (e.g., VEGF). Trx has been shown to increase hypoxia-inducible factor 1 α protein expression associated with increased vascular endothelial growth factor production and enhanced tumor angiogenesis [28]. Thus, Trx may act at several levels- including as a scavenger of ROS, as a cytokine and as an inducer via HIF-1 of tumor angiogenesis- to promote cancer cell proliferation and resistance of tumors to therapy.

Thioredoxin Binding Protein (TBP-2)- Thioredoxin (Trx) Interactions

Human Trx- Binding Protein (TBP-2) was identified using a yeast two-hybrid assay and designated Trx-binding protein-2 [29]. Data base analysis revealed that TBP-2 is identical to the previously reported vitamin D3 up-regulated protein-1 [30]. It has been shown that TBP-2 interacts with Trx (29,31). TBP-2 stably bound Trx, *in vitro* or *in vivo*, has an inhibitory effect on Trx dependent reducing activity. Thus, TBP-2 is a negative regulator of Trx (Figure 1B)

TBP-2 is expressed at low levels in many human breast cancers, colon cancers [32], gastrointestinal cancer [33], malignant pheochromocytomas [34], human T-cell leukemia [26] and high grade B-cell lymphoma [35]. It is also reported to be down-regulated in high fat induced mouse breast cancer [36] and carcinogen induced rat renal carcinoma [37].

A decrease in TBP-2 is associated with high proliferation of aortic smooth muscle induced by over expression of Trx [38]. Many stress stimuli can induce increased expression of TBP-2, including H₂O₂, TGF- β and UV [39], histone deacetylase inhibitors [32], 5 fluorouracil [40], heart shock [41] and carcinogens [42]. Over expression of TBP-2 can be associated with inhibition of cell growth [32]. HDACi can induce increased expression of TBP-2 which is associated with inhibition of transformed cell growth (see below) [32]. High levels of expression of TBP-2 can block cell-cycle progression, with arrest of cells in G1 and inhibit colony formation of transformed cells [43,44].

Histone Deacetylases: (HDACs)

The genetic code for proteins resides in the base sequences of DNA, while the expression of genes is largely regulated by the structure of chromatin (epigenetic gene regulation), which is a complex of DNA, histones and non-histone proteins [45-47]. Nucleosomes, the basic repeating units of chromatin, have a core histone octamer composed of two of each of four histones: H2A, -2B and -3 and -4 with the DNA wrapped around the core. Chromatin remodeling involves reversible post-translational modification of amino acids in the histone

tails by acetylation of lysines, methylation of lysines and arginines, phosphorylation of histidines, serines and threonines, ubiquitination and sumoylation of lysines and ADP-ribosylation of glutamic acid (48-50). Two groups of enzymes, histone deacetylases (HDACs) and histone acetyl transferases (HATs), primarily determine the pattern of histone acetylation. It has been hypothesized that histone modifications acting alone, sequentially or in combination, represent a 'code' that can be recognized by non-histone proteins forming complexes that are involved in the regulation of gene expression [48].

In humans, 18 HDAC enzymes that have been identified and categorized into three classes based on their homology to yeast HDACs [51-55]. Class I includes HDAC1, -2, -3 and -8, which are related to the yeast histone deacetylase gene RPD3, with molecular weights of 22-55kDa and share homology in their catalytic sites. Class II includes HDAC4, -5, -7 and -9, which are larger molecules with molecular weights between 120 and 135 kDa, are related to yeast HDA1 deacetylase. A subclass of HDACs is represented by HDAC6 and -10, which contain two catalytic sites. Both class I and II HDACs are zinc-binding enzymes and are inhibited by a variety of compounds, such as the hydroxamic acids, trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (56). A third class of HDACs has been identified that has an absolute requirement for NAD, the so-called Sir2 family of deacetylases, which are not inhibited by compounds, such as TSA or SAHA.

There is abundant evidence that HDACs are not redundant in their biological activity. Class I HDACs are primarily present in the nucleus and are ubiquitous, whereas class II HDAC expression is more tissue restricted and shuttle between the nucleus and cytoplasm. Class I HDACs play a role in cell death and proliferation pathways whereas class II HDACs appear to be important for tissue-specific functions.

Many proteins in addition to histone are substrates for HDACs and HATs deacetylation and acetylation- with consequent alteration in structure and function of these proteins. For example, these proteins include oncoprotein (Bcl-6), tumor suppressors (p53), transcription factors (NF- κ B, E2F.1, YY-1, TFIIIB) structural proteins (tubulin), and chaperone proteins (Hsp90)(47, 51)

Histone Deacetylase Inhibitors (HDACi)

HDACi are a promising group of anti-cancer agents that induce cell death of a broad spectrum of transformed cells, whereas normal cells are relatively resistant to these agents [32,54, 57-63]. HDACi that have been reported to date can be divided into several chemical structural classes including hydroxamates, cyclic peptides, aliphatic acids and benzamides [59]. TSA was the first natural product hydroxamates discovered to inhibit HDACs. SAHA is structurally similar to TSA and a nanomolar inhibitor of partially purified HDAC of class I and II (56, 64). The selective effect of HDACi, such as SAHA, in causing transformed but not normal cell death is not due to differences between transformed and normal cells in the accumulation of acetylated histones.

It has been shown that HDACi such as SAHA and TSA interact directly with the catalytic site of the enzyme, the hydroxamic acid moiety binding to the zinc molecule at the base of the catalytic pocket, inhibiting the activity of the enzymes (Figure 2) (65,66). SAHA inhibits the growth of a broad spectrum of hematologic and solid tumors both *in vitro* (cell cultures) and *in vivo* in animal models [67]. In phase I and II clinical trial, SAHA has had significant anti-cancer activity in patients with both hematologic and solid tumors at well tolerated doses [62,68,69].

Effects of Histone Deacetylase Inhibitors

The mechanisms of the growth arrest and death of cells caused by HDACi is not completely understood [70]. These inhibitors cause the accumulation of acetylated forms of histone and non-histone proteins substrates that are involved in the regulation of gene expression, cell proliferation, cell migration, angiogenesis, and cell death. Defining the role of these different targets is important in understanding the anti-cancer activity of HDACi.

It has been shown that HDACi selectively alter gene expression [55,71-73]. Microarray analysis of the effects of HDACi on gene expression in different cancer cell lines has shown that the patterns of alteration of gene expression are similar for different HDACi, as well as showing definite differences induced by different agents in various transformed cells [74]. Almost an equal number of genes were increased in expression as were repressed. SAHA is a potent inducer of apoptosis of human multiple myeloma cells. Microarray analysis of gene expression in these cells revealed that a constellation of antiproliferative and/or proapoptotic genes was altered within 6 hrs of culture with SAHA, including down-regulation of transcripts of a member of the insulin-like growth factor (IGF)/IGF-1 receptor and IL-6 receptor signaling cascades and antiapoptotic genes, such as caspase inhibitors, oncogenic kinases, DNA synthesis repair enzymes and transcription factors, such as E2F-1 [75]. SAHA also suppressed the activity of the proteasome and expression of its subunits, and enhanced multiple myeloma cell sensitivity to proteasome inhibition and other proapoptotic agents. Among the other genes that are commonly repressed by HDACi are cyclin D1, erb-B₂ and thymidylate synthase. One gene whose expression is increased by SAHA in almost all the transformed and normal cells examined is TBP-2. (see below)

The underlining mechanism for this selectivity in altering gene expression is not known. Studies using DNA arrays showed only 2-10% of the genes that were altered in their expression with about equal number decreased as measured in various transformed cells treated with TSA or SAHA [72,76-79]. The CDK inhibitor p21^{waf}, which can lead to the arrest of cells in G1, is one of the most commonly induced genes. The p21 gene promoter has been shown to be a direct target for SAHA [78,86]. It was found that in ARP-1 cells (human multiple myeloma), SAHA causes specific modifications in the pattern of acetylation and methylation of lysines in histones H3 and -4 associated with the proximal promoter of the p21 gene which did not occur in promoter associated histones of genes whose expression was not altered. SAHA caused a marked decrease in HDAC1 and myc and recruited RNA polymerase II to the protein complex bound to the p21 promoter. These changes in the protein composition of the transcription factor complex are consistent with an increased expression of the p21 gene observed in cells treated with SAHA. As reviewed below, SAHA directly acts on the promoter region of the TBP-2 gene as well, associated with increased expression of this gene. The changes induced in the TBP-2 promoter associated transcription factor proteins, if any, have not been defined.

Effects of Histone Deacetylase Inhibitors on TBP-2 and Trx

SAHA, as other HDACi, and various anti-cancer agents cause an increase in reactive oxygen species (ROS) in transformed but not in normal cells [61,81,82,84,85]. The best defined programmed cell death is mitochondrial mediated caspase dependent apoptosis. Apoptosis in mammalian cells is mediated by the family of cysteine proteases, the caspases [61,86-88]. Transformed cells but not normal cells cultured with SAHA accumulate ROS and have a marked increased in caspase activity. Caspase activation via the cytochrome *c* mitochondrial pathway can be induced by the production of ROS. A striking finding is that SAHA-induced death of many transformed cells is blocked very little or not at all by inhibiting the activation of the caspase pathway [61,89,90,91]. Thus, HDACi can induce both caspase dependent apoptosis and caspase-independent cell death. The caspase-independent cell death is associated

with increased levels of TBP-2, and presumably, inhibition of the reducing activity of Trx [32].

TBP2- is ubiquitously expressed in normal human tissue [32]. The level of expression of this protein is relatively high in kidney, skeletal muscle and spleen and relatively low in brain. By comparison the expression of TBP-2 is low in several human cancers, including breast [32], colon [32], T-Cell leukemia virus type I [26], and renal cell carcinoma [37].

TBP-2 and Trx appear to play an important role in HDACi induced caspase-independent transformed cell death [58,81]. Using microarray analysis, it was found that SAHA increases the level of TBP-2 mRNA in a number of human transformed cell lines, including prostate carcinoma, bladder carcinoma, myeloma, and breast cancer [32] (Figure 1). The effect of SAHA in increasing TBP-2 expression is both concentration and time dependent. In LNCaP cells, a human prostate cancer cell, 7.5 μ M, SAHA increased TBP-2 mRNA levels within 30 min of culture [32]. In these cell lines, SAHA induced growth arrest and/or cell death at concentrations that induced TBP-2 mRNA. The induction of TBP-2 was associated with a decrease in Trx levels consistent with the fact that TBP-2 can bind Trx and inhibit its reducing activity [26,32]. Supporting the important role of inactivation of Trx in the sensitivity of certain cancer cells to HDACi, is the fact that decreasing Trx levels with siRNA for Trx significantly increased the sensitivity of the cells to SAHA [81].

By contrast to the effect of SAHA on Trx protein levels and Trx activity in transformed cells, HDACi increased Trx in normal cells in culture [26,30]. The mechanism of SAHA induced increased levels of Trx in normal cells is not understood. Nevertheless, it can account, in part at least, for the relative resistance of normal cells to HDACi induced increase in ROS and cell death. Consistent with this hypothesis is the finding that the free radical scavenger, N-acetyl-L-cysteine accentuates HDACi mediated ROS generation and cell death of cells in culture [92].

A recent study found that malignant mesothelioma (MM) cells express Trx protein at low levels compared to its non-malignant counterpart cells [25], while Trx mRNA levels were comparable. α -tocopheryl succinate (α -TOS) and TNF-related apoptosis- inducing ligand (TRAIL) induced apoptosis in MM cells but not the normal counterpart cells. α -TOS induces ROS dependent cell death, while TRAIL induced primarily caspase mediated apoptosis. Down-regulation of Trx sensitized the non-malignant cells to TRAIL, but not α -TOS despite the increase in ROS. A finding in MM which is different from that observed in other transformed cells, as indicated above. These findings suggest that the effect of agents such as HDACi in inducing the accumulation of ROS and cell death is determined by cell "context" and the molecular basis of such differences is not yet defined. The mechanism by which SAHA induces the expression of TBP-2 mRNA has been shown to involve a direct action on the TBP-2 promoter, as shown in the following studies: the TBP-2 promoter region was cloned [32]. The promoter sequence was analyzed for the presence of classical promoter features. The presence of a putative TATA box as well as putative binding sites for the transcription factors, such as, NF-Y, Myc-Max, E2F, vitamin D receptor-retinoid X receptor, and NF- κ B were identified (Figure 3A) [32]. This sequence of DNA was shown to have promoter activity in luciferase assays.

To further test whether SAHA induces the activity of the cloned TBP-2 promoter, 293T cells were transfected with reporter constructs and cultured with the HDACi. The activity of the TBP-2 promoter fragment was induced in a dose-dependent manner by SAHA (Figure 3D). The activity of the TBP-2 promoter was also induced by *m*-carboxycinnamic acid bishydroxamic acid (CBHA) a related hydroxamic acid -based hybrid polar inhibitor of HDAC activity. Further, it was found that a site between promoter constructs -482 and -440 is critical

for optimal induction of TBP-2 by HDACi. This region of the promoter contains putative E box and inverted CCAAT box sites. Several transcription factor, including NF-Y [93], bind to the inverted CCAAT box. Introduction of two point mutations into the inverted CCAAT box (ATTGG→AGTAG) markedly decreased the level of induction by SAHA (Figure 3D). These findings indicate that the inverted CCAAT box in the TBP-2 promoter is critical for the optimal induction of the TBP-2 promoter by SAHA. Taken together, these results support a role for NF-Y and the CCAAT box in the promoter induction of TBP-2 transcription by the direct action of SAHA on this gene. The fact that HDACi can induce increased expression of the TBP-2 gene in cancer cells maybe an important factor in cell death induced by these agents since TBP-2 binds to and blocks Trx activity.

Summary and Conclusions

HDACi are promising new anti-cancer agents. These agents can cause transformed cells to undergo growth arrest and cell death. SAHA is an HDACi in phase I and II clinical trials which has shown significant anti-cancer activity in patients with both hematologic and solid tumors at well tolerated doses. SAHA can induce caspase dependent apoptosis and caspase independent cell death. The caspase independent cell death is associated with SAHA induced accumulation of ROS. Trx is the major intracellular scavenger of ROS. High levels of Trx are associated with resistance of cancer cells to HDACi (and other anti-cancer agents). Normal cells are relatively resistant to HDACi induced cell death and high levels of Trx may account, in part, for this resistance.

TBP-2 binds to Trx and inactivates the reducing activity of this protein. Many cancers have low levels of expression of TBP-2. SAHA induced an increased expression of TBP-2 in transformed cells. An increase in TBP-2 associated with decrease in Trx may play an important role in HDACi induced cancer cell death. These findings have important implications for cancer therapy- making the development of tumor cell selective inducers of TBP-2 an important strategy for improving treatment of malignant diseases.

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Thioredoxin-redox system and histone deacetylase inhibitor action: **(A)** model for the effect of histone deacetylase inhibitor (HDACi) in normal cells. HDACi increases the level of reduced thioredoxin (Trx-SH-SH) which act to scavenger free radical oxygen species (ROS). Trx-SH-SH has several targets including ribonucleotide reductase required DNA synthesis, transcription factors, such as NF- κ B and receptors such as the estrogen receptor (ER). **B**: Model for the effect of HDACi in transformed cells. HDACi induced thioredoxin binding protein (TBP-2) which binds to and inactivates TRX-SH-SH, and, inturn, facilitates apoptosis or ROS induced cell death. (adapted from Figure 1, Reference 70)

SuberoylAnilide Hydroxamic Acid

Figure 2.

A) Structure of suberoylanilide hydroxamic acid (SAHA)

B) Schematic representation of structure of catalytic site of histone deacetylase protein with SAHA (see reference 65)

Figure 3.

A: Schematic representation of TBP2 promoter. B: Schematic representation of the putative TBP-2 promoter region and the deletion mutants. The position of putative transcription factors binding sites in the promoter are shown: 1, NF- κ B binding site; 2, vitamin D receptor/retinoid X receptor responsive element; 3, E2F binding site; 4, E box; 5, inverted CCAAT box; 6, CCAAT box; 7, E box; 8, TATA box. C: Activity of deletion mutants. Different lengths of the 5' flanking region of human TBP-2 gene were amplified by PCR and cloned upstream of the luciferase gene in the pGL-2 vector. The constructs were transfected into 293T cells and luciferase activity was measured. D: SAHA induction of deletion mutants. Parallel experiments were carried out as in C except SAHA was added 12 h after transfection. Luciferase activity was normalized against total protein. D: The inverted CCAAT box is critical for SAHA inducibility. PCR-based mutagenesis was performed to mutate the inverted CCAAT box in the isolated TBP-2 promoter. The mutant promoter was then cloned into pGL-2 and transfected into 293T cells with or without SAHA (2 μ M) treatment. The TBP-2 promoter construct with the mutations in the inverted CCAAT box has about 8% of wild-type promoter activity (not shown). Results shown are the average of three independent transfection $\pm$ SD. (adapted from reference 32)