Histone deacetylase inhibitors regulate p21^{WAF1} gene expression at the post-transcriptional level in HepG2 cells

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Abstract Histone deacetylase inhibitors (HDIs) are thought to act primarily at the level of transcription inducing cell cycle arrest, differentiation and/or apoptosis in many cancer cell types. Induction of the potent cdk/cyclin inhibitor p21^{WAF1} is a key feature of this HDI mediated transcriptional re-programming phenomenon. However, in the current study we report that HDIs are also capable of inducing p21^{WAF1} through purely post-transcriptional events, namely increased mRNA stability. These studies highlight our growing appreciation for the complexities of HDI mediated effects and challenge our preconceptions regarding the action of these promising anti-neoplastics.

Keywords: mRNA stability; Histone deacetylase inhibitor; p21^{WAF1}

1. Introduction

A number of histone deacetylase inhibitors (HDIs) are being analyzed at the clinical level [1–4]. However, it is not precisely understood how HDIs elicit their anti-neoplastic effects. Hypotheses have been generated which suggest that HDIs are transcriptional activators that disrupt the balance between histone deacetylase (HDAC) and histone acetyl transferase (HAT) activity resulting in hyperacetylation of nucleosomal histones and non-histone proteins, such as the transcription factor p53 [5,6]. The cyclin-dependent kinase inhibitor, p21^{WAF1}, is the most extensively studied HDI responsive gene and the only known gene whose activation has been linked to HDI mediated cell cycle arrest [7]. Gene expression of p21^{WAF1} is directly upregulated by HDIs in a p53 independent fashion, but requires one or more Sp1 binding sites in the p21^{WAF1} proximal promoter for transcriptional activation by these agents [8,9]. Furthermore, p21^{WAF1} fits the classical model of HDI mediated induction, as acetylation of core promoter histones H3 and H4 correlates with increased gene expression [10]. However, recent evidence implies that the phosphatidylinositol 3-kinase and protein kinase C epsilon signaling pathway is required in addition to increased histone acetylation for p21^{WAF1} activation [9,11]. Moreover, HDIs regulate only 2–5% of cellular genes and have also been reported to repress a number of genes, including the proto-oncogene c-myc and the colon oncogene c-Src [12,13]. Therefore, these more recent observations suggest that histone hyperacetylation may not be the only fundamental mechanism underlying HDI mediated changes in gene expression. Here we report that in a human hepatocellular carcinoma cell line, HepG2, HDIs are incapable of directly activating the p21^{WAF1} promoter. Instead, the HDI mediated induction of p21^{WAF1} mRNA and protein results from a dramatic increase in p21^{WAF1} mRNA stability. These findings further challenge the preconception that HDIs solely regulate changes in gene expression by histone acetylation and transcriptional activation.

2. Materials and methods

2.1. Cell culture and treatments

The cell lines utilized in these experiments were obtained from the American Type Culture Collection (ATCC). The HT29 colon carcinoma cells were maintained in Dulbecco's modified Eagle's medium. HepG2, hepatocellular carcinoma cells were grown in Dulbecco's modified Eagle's medium and Ham's F-12 medium. All cells were supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc.) and 1% penicillin/streptomycin (Gibco) and grown at 37 °C and 5% CO_2. For HDI treatments, 5 mM sodium butyrate (NaB, Sigma), or 1 μM trichostatin A (TSA, Sigma) was added directly to the media of cells grown to approximately 50% confluency, and harvested at various time points. Similarly, cells were treated with 50 μM Cycloheximide (CHX, Sigma) in the absence or presence of HDIs. For mRNA half-life studies, 5 μg/ml Actinomycin D (Act. D, Sigma) was added directly to the media of cells grown to 50% confluency.

2.2. Plasmid constructs

The human wild-type WAF1 promoter–luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University). The 2.3 kb WAF1 promoter fragment was isolated by *Hin*dIII digestion and subcloned into the *Hin*dIII site of the pBlue vector (Stratagene). The −210WAF1 promoter was constructed by *Pst*I digestion and re-ligation prior to removal from pBlue with *Sac*I and cloning into *Sac*I digested pCAT3-Basic (Promega). The −101WAF1 CAT promoter construct was designed by introducing a *Sac*I site through site directed mutagenesis, using the mutagenic primers sense: 5′GGGCGGTCCCGGGCGGAGCTCTGGGCCGAGCGAGGGTCCC3′ and antisense: 5′GGGAC CCGCGCTCGGCCCAGAGCTCCGCCCGGGACCGCCC3′. The

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Abbreviations: HDI, histone deacetylase inhibitor; NaB, sodium butyrate; TSA, trichostatin A; HDAC, histone deacetylase; HAT, histone acetyl transferase; CHX, cycloheximide; Act. D, actinomycin D; CAT, chloramphenicol acetyl transferase

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mutagenic construct was digested with *Sac*I and re-ligated to generate −101WAF1 CAT.

2.3. Growth curve experiments

HepG2 cells were seeded at 1.0×10^5 cells per 35 mm tissue culture plate and allowed to grow under normal conditions for a 48 h time period. At this time the media was changed and the cells treated with different doses of NaB (mM) or TSA (μM). Cells were trypsinized and counted with a Coulter Counter ZM (Coulter Electronics, Inc.) 24, 48, 72, and 96 h following treatment. The growth curve data presented is the result of at least two independent experiments.

2.4. RNA extraction and northern blot analysis

Total RNA was isolated from cells by the guanidinium thiocyanate method outlined by Chomczynski and Sacchi [14] and resuspended in 0.1 mM EDTA containing diethyl pyrocarbonate (DEPC) water. Fifteen microgram samples of total RNA were separated on a 1% denaturing formaldehyde-agarose gels, transferred to GeneScreen Plus hybridization transfer membranes (Perkin–Elmer Sciences) and UV cross-linked. cDNA probes as previously described [13] were randomly labeled with [α-^{32}P]dCTP using Ready-To-Go DNA Labelling Beads (Amersham Biosciences). Images were acquired by use of a Bio-Rad Molecular Imager FX following exposure to an Imaging Screen K (Kodak) or by autoradiography at −80 °C with the aid of an intensifier screen (Kodak).

2.5. Immunoblot analysis

Cells were harvested following HDI treatment at selected time points in an SDS sample buffer containing 10% (w/v) glycerol, 5% β-mercaptoethanol, 2% (w/v) sodium dodecyl hydrogen sulfate, 65 mM Tris, and 0.05% (w/v) bromophenol blue to pH 6.8. A Lowry Assay (Sigma) was used to determine protein concentration and 30 μg of protein sample was resolved on a 10% SDS–polyacrylamide gel. Following electrophoresis, the protein was transferred to Optitran Supported Nitrocellulose (Schleicher & Schuell) and blocked using standard procedures [13]. The membrane was incubated with p21 (187) sc-817 mouse monoclonal antibody (Santa Cruz Biotechnology) at a final concentration of 200 ng/ml, washed and incubated for a second time with anti-mouse horseradish peroxidase (Santa Cruz Biotechnology) antibody at 100 ng/ml, which was diluted 1:2000. A second wash was performed prior to blot treatment with Chemiluminescence Reagents (Perkin–Elmer Life Sciences) and exposure to X-OMAT Blue XB-1 film (Kodak) for detection.

2.6. Transient transfections and CAT assays

Plasmid constructs were isolated and purified with an EndoFree Plasmid Maxi Kit (Qiagen). 1.0 μg of WAF1 promoter CAT construct, 2.0 μg of pBlue, 1.0 μg CMV β-Gal, and 85 μl of serum-free DMEM were mixed together with 10 μl of superfect (Qiagen) and incubated for 20 min at room temperature. The transfection mix was diluted further with 600 μl DMEM containing 10% FCS and added to directly to HT29 or HepG2 cells seeded the previous day at 3×10^6 cells per 35 mm tissue culture plate. Similarly, a transfection mixture containing 1.0 μg of WAF1 promoter CAT construct, 1.0 μg of pBlue, 1.0 μg CMV β-Gal, and 1.0 μg of CMV c-jun (purchased from ATCC) was prepared. Transfections were carried out as previously described [15]. The data presented are the result of at least two experiments, each of which were performed in duplicate.

3. Results

Various classes of HDIs have been shown to harbor potent anti-tumor activities in a range of human tumor cells and animal models, indicating that these agents may have promising therapeutic value [3,4]. NaB was one of the first HDIs to be clinically investigated and belongs to the class of short chain fatty acids. This agent is a non-competitive HDI that exists at physiologically relevant concentrations within the colon as a byproduct of anaerobic bacterial fermentation of dietary fiber [5,16]. TSA is a much more potent HDI and belongs to the group of hydroxamic acids [17]. Although, structurally dissimilar, both NaB and TSA are capable of promoting cell cycle arrest, differentiation, and/or apoptosis in tumor cells, where induction of p21^{WAF1} by HDIs appears to be important for suppression of cellular proliferation [7]. To investigate whether HDIs had anti-proliferative effects in the HepG2 cells, these hepatocellular carcinoma cells were cultured alone or with different concentrations of NaB or TSA for 24, 48, 72, and 96 h. Growth curve analyses indicated that NaB decreased cell growth in a dose dependent manner. A dose of 1.0 mM NaB was capable of inhibiting cellular proliferation and 2.0 mM NaB was optimal for preventing growth of HepG2 cells (Fig. 1A). TSA, on the other hand, inhibited cell growth at significantly lower concentrations, where 1.0 μM TSA was sufficient for the complete absence of HepG2 cellular proliferation (Fig. 1B). Trypan blue staining confirmed the viability of HepG2 cells following 96 h of HDI treatment at the optimal doses.

Given that HDIs appear to be effective anti-proliferative agents in HepG2 cells, we investigated whether p21^{WAF1} expression was upregulated by HDIs independent of de novo protein synthesis, similar to a number of previously documented examples [8,13]. Time course studies performed in the presence of NaB (5 mM) indicated a dramatic increase in the p21^{WAF1} levels in a time dependent manner, as we have previously observed (Fig. 2A, top left panel and B) [13]. Furthermore, TSA (1 μM), was also capable of increasing p21^{WAF1} protein expression (Fig. 2C). However, we were surprised to find that NaB mediated induction of p21^{WAF1} was blocked in the presence of a protein synthesis inhibitor, Cycloheximide (Fig. 2A, top right panel). Northern blots re-probed with c-Src indicated that NaB was capable of repressing the mRNA expression of this oncogene independent of new protein synthesis (Fig. 2A, bottom panels) [13]. Cumulatively, these observations suggest that p21^{WAF1} may be an indirect target of HDIs in HepG2 cells. To our knowledge this is the first example where protein neo synthesis is required for HDI mediated p21^{WAF1} induction.

Since NaB appears to stimulate p21^{WAF1} gene expression dependent on de novo protein synthesis, we next inquired whether the WAF1 promoter could still be activated by HDIs. Transient transfection assays were performed in HepG2 cells with WAF1 CAT promoter constructs in the absence or presence of HDIs. Several reports have shown that induction of p21^{WAF1} by HDIs occurs independently of p53, but requires one or more Sp1 binding sites located in the proximal promoter [7–9]. Here we examined two different WAF1 CAT promoter constructs to determine if the WAF1 promoter could be activated by HDIs in HepG2 cells. These constructs have previously been shown to be inducible by HDIs in a number of reports [7–9]. Cells were transiently transfected with WAF1 CAT promoter constructs and treated with either NaB or TSA. Interestingly, we observed that neither the −210WAF1 CAT or −101WAF1 CAT promoter constructs could be activated in HepG2 cells by TSA, and were only marginally upregulated by NaB (Fig. 3C). A larger construct, −2300WAF1 CAT, which harbours two p53 response elements was similarly unresponsive to the presences of HDIs in HepG2 cells (results not shown). In contrast, transient transfections performed in HT29 cells verified that the WAF1 promoter constructs were activated as expected. In these cells the promoter activity of −210WAF1 CAT was increased 15- and 30-fold, respectively, by NaB and TSA. Similarly, the −101WAF1 CAT promoter construct was activated approximately 6- and 4-fold by NaB and TSA respectively (Fig. 3B). Since p21^{WAF1} is constitutively expressed at low levels in HepG2

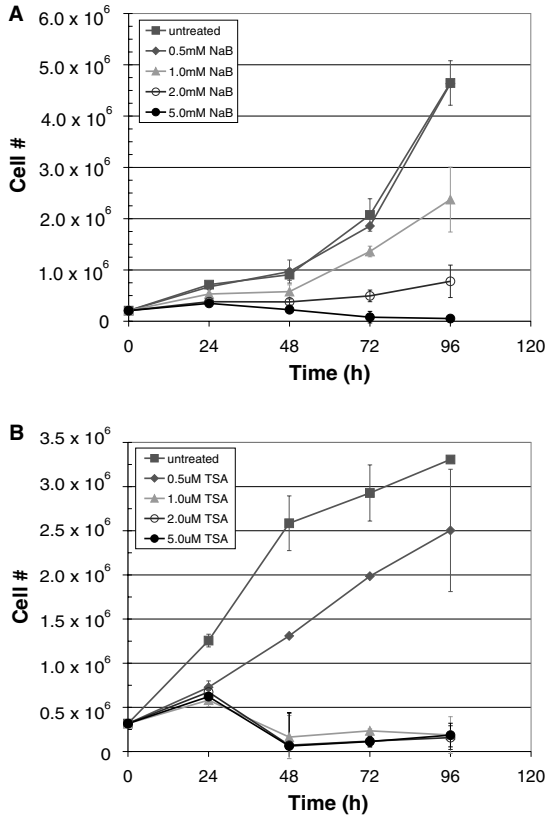

Fig. 1. HDIs decrease cellular proliferation in HepG2 cells. Growth curve analysis of HepG2 cells counted following treatment with increasing concentrations of NaB (A) or TSA (B). Data represent the average of two independent experiments and includes standard deviations.

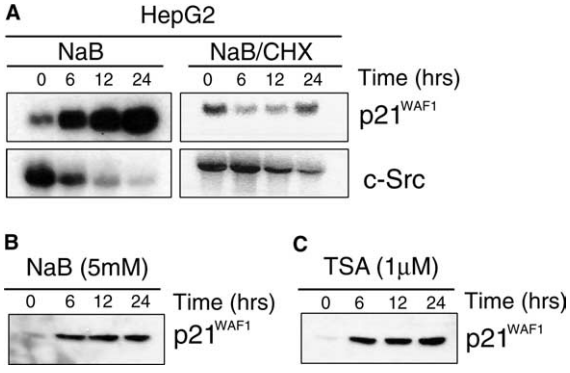

Fig. 2. HDI upregulation of $p21^{WAF1}$ gene expression in HepG2 cells requires protein neo synthesis. (A) Total RNA was extracted from HepG2 cells following various periods of exposure to NaB (5 mM), CHX (50 μM), or a combination of the two. RNA was examined by Northern blot analysis for $p21^{WAF1}$ and c-Src. (B) Total cellular extracts were isolated from HepG2 cells following incubation with NaB (5 mM) or TSA (1 μM) for varying time points and analyzed by Western blot for $p21^{WAF1}$ protein expression.

cells we felt it important to determine if the promoter was actually capable of any supplemental transcriptional activation. Co-transfection experiments were therefore performed with the WAF1 promoter constructs in the presence of a known $p21^{WAF1}$ trans-activator, c-jun [18]. Co-transfection data confirmed that the WAF1 promoter was capable of further activation, being upregulated by c-jun in HepG2 cells approximately 2.5- and 6.0-fold in −210WAF1 CAT and −101WAF1 CAT activity, respectively (Fig. 3C). Lastly, nuclear run-on assays suggested that the rate of WAF1 transcription did not increase following NaB or TSA treatment in HepG2 cells (results not shown). Therefore, these observations show that the WAF1 promoter is unresponsive to HDIs, and that post-transcriptional mechanisms must be responsible for the observed induction of $p21^{WAF1}$ expression in HepG2 cells.

We next performed a series of mRNA half-life studies in HepG2 cells. Act. D was added to HepG2 cells to prevent transcription followed by a 12 h incubation period in the presence or absence of HDIs. Northern blot analysis was performed on the mRNA samples and $p21^{WAF1}$ mRNA levels were examined (Fig. 4A, top panels). Ribosomal protein PO (RPPO) was used as a control to normalize the data (Fig. 4A, bottom panels). The half-life of $p21^{WAF1}$ mRNA in control HepG2 cells was calculated to be approximately 85 min. However, in HepG2 cells treated with NaB or TSA the $p21^{WAF1}$ mRNA half-life increased approximately 3.1-fold (270 min) and 5.4-fold (460 min), respectively (Fig. 4B). Taken together, our data show that the HDIs, NaB and TSA upregulate $p21^{WAF1}$ mRNA and protein levels by post-transcriptional mechanisms in HepG2 cells. These results represent the first reported example of HDI mediated mRNA stabilization.

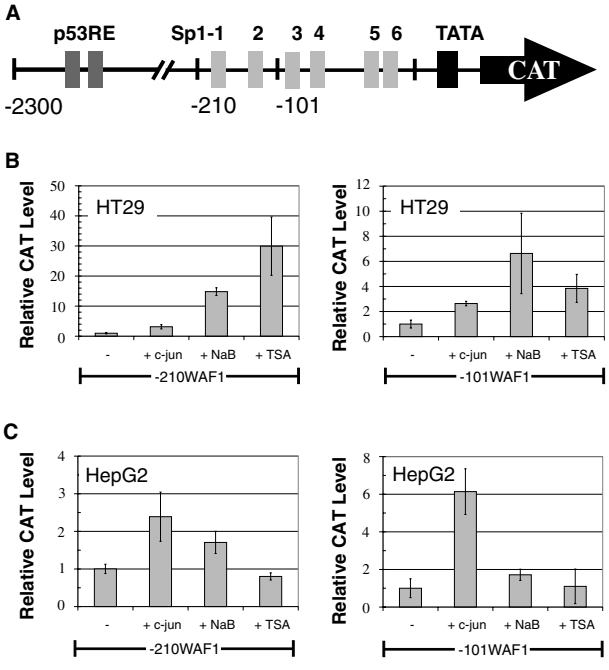

Fig. 3. The WAF1 promoter is not activated by HDIs in HepG2 cells. (A) Diagram representing various WAF1 CAT constructs used in this study showing the positions of critical Sp1 sites. HT29 (B) or HepG2 (C) cells were transiently transfected with WAF1 CAT promoter constructs; −210WAF1 CAT and −101WAF1 CAT. (B, C) Co-transfections were performed with the WAF1 promoter constructs and exposed to NaB (5 mM), TSA (1 μM), or left untreated. A second set of co-transfections were performed with the WAF1 promoter constructs and c-jun. The CAT levels were determined relative to the untreated WAF1 co-transfected HT29 (B) and HepG2 cells (C). Results represent two independent experiments performed in duplicate.

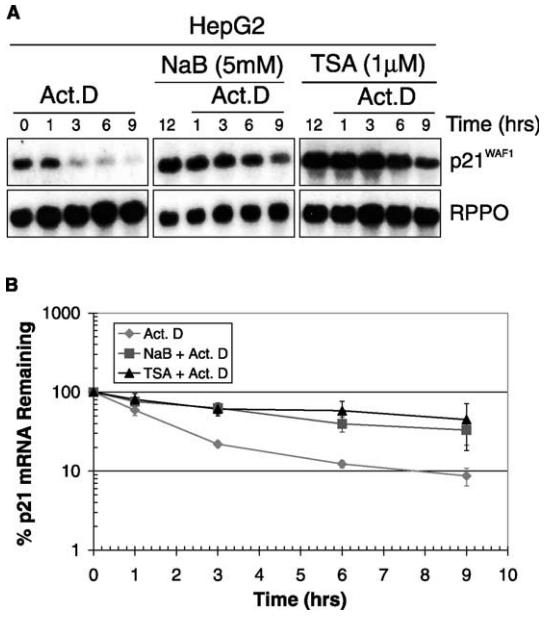

Fig. 4. HDIs increase the stability of $p21^{WAF1}$ mRNA in HepG2 cells. (A) Total RNA was isolated from HepG2 cells treated with Act. D in the presence or absence of NaB (5 mM) or TSA (1 μM) and examined by Northern blot analysis for $p21^{WAF1}$ and RPPO. (B) The $p21^{WAF1}$ mRNA signal was determined by a Molecular Imager and normalized to the RPPO mRNA signal. Data were plotted on a semi-logarithmic scale and represent means ± the standard errors of the means of two independent experiments.

4. Discussion

HDIs represent a diverse array of structurally distinct compounds that are being extensively studied for their chemopreventative and therapeutic activities in a number of neoplasias. While HDIs are known to cause cell cycle arrest, differentiation and/or apoptosis the mechanisms associated with these antitumor activities have yet to be uncovered. Characteristically, HDIs have been documented to be transcriptional activators, although more recent accounts have indicated that HDIs do not solely alter chromatin dynamics to stimulate gene transcription but rather appear to be capable of much more diverse events. For instance, HDIs have been implicated in the acetylation of numerous non-histone proteins including the transcription factors, p53 and E2F1 [6]. Furthermore, the mechanisms responsible for HDI mediated repression of gene expression have yet to be elucidated [12,13]. Here we have demonstrated for the first time that HDIs are also capable of regulating gene expression levels by post-transcriptional mechanisms. In this study, we determined that treatment of HepG2 cells with HDIs resulted in an impressive induction of $p21^{WAF1}$ mRNA and protein. However, this induction was not the result of transcriptional activation and required protein neo-synthesis, in contrast to numerous reports in other cell lines. These results were confirmed in transfection studies with various WAF1 promoter constructs, including the full-length, −2300 WAF1 CAT, construct. Although we noted a very minor NaB mediated increase in WAF1 promoter activity this was not seen with the more specific HDI, TSA. However, both NaB and TSA dramatically enhanced $p21^{WAF1}$ mRNA stability and produced an approximately 3.1- and 5.4-fold increase, respectively, in HepG2 cells, indicating that post-transcriptional events play a dominant role in HDI mediated upregulation of $p21^{WAF1}$ in HepG2 cells. Stabilization of $p21^{WAF1}$ mRNA has been noted before. For example, Elav-like proteins binding to AU-rich region of the 3′ non-coding region have been implicated in UVC mediated stability of $p21^{WAF1}$ mRNA [19]. However, we have determined that the AU-rich region of the $p21^{WAF1}$ mRNA does not display increased binding following HDI treatment (results not shown). Therefore, it is unlikely that Elav-like proteins are responsible for HDI mediated upregulation of the $p21^{WAF1}$ mRNA in HepG2 cells. Clearly further work will be required to determine the mechanism of HDI mediated mRNA stabilization.

Taken together these observations suggest that HDIs have the potential to influence $p21^{WAF1}$ gene expression levels independent of chromatin remodeling and transcriptional activation, at least in some cell lines. Furthermore, HDI mediated changes in the mRNA stability may be more universal, and function in collaboration with histone acetylation to influence changes in gene expression. Although the work described here relates to a single cell line future work regarding such HDI mediated changes in gene expression must take these observations into account.

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