Histone Deacetylase Inhibitors All Induce p21 but Differentially Cause Tubulin Acetylation, Mitotic Arrest, and Cytotoxicity

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Abstract

By preventing deacetylation of histones, histone deacetylase inhibitors (HDIs) transcriptionally induce p21. Here we show that the HDIs sodium butyrate (Bu), trichostatin A (TSA) and depsipeptide (FR901228) all induced p21, but only TSA and FR901228 caused mitotic arrest (in addition to arrest in G_1 and G_2). The ability to cause mitotic arrest correlated with the higher cytotoxicity of these compounds. Although causing mitotic arrest, TSA and FR901228 (unlike paclitaxel) did not affect tubulin polymerization. Unlike FR9012208, TSA caused acetylation of tubulin at lysine 40; both soluble tubulin and microtubules were acetylated. Whereas the induction of p21 reached a maximum by 8 h, tubulin was maximally acetylated after only 1 h of TSA treatment. Tubulin acetylation was detectable after treatment with 12–25 ng/ml TSA although acetylation plateaued at 50 ng/ml TSA, coinciding with G_2-M arrest, appearance of cells with a sub-2N DNA content, poly(ADP-ribose) polymerase cleavage, and rapid cell death. We conclude that HDIs have differential effects on non-histone deacetylases and that rapid acetylation of tubulin caused by TSA is a marker of nontranscriptional effects of TSA.

Introduction

HDACs[2] is a class of enzymes consisting of at least two subfamilies with at least eight members (1, 2). HDIs are currently undergoing clinical trials (2). The inhibition of HDAC in cancer cells can lead to transcriptional modulation of 1–2% of genes (1, 3). HDIs including butyrate, TSA, oxamflatin, suberoylanilide hydroxamic acid and FR901228 induce p21 (4–10). HCT116 cells lacking p21 do not undergo G_1 arrest, continue DNA synthesis, and arrest in G_2-M phase of the cell cycle (11). However, p21 can protect against apoptosis (11, 12). In leukemia cells, butyrate and other HDIs caused G_2-M cell cycle arrest and apoptosis (13). Forced G_0-G_1 arrest by p16 protected the cells from butyrate-induced cell death without affecting the extent of histone acetylation, which suggests that the latter may not be sufficient for cell death (14). Apoptosis, but not arrest, was delayed by the caspase inhibitor zVAD and to a lesser extent by DEVD and VEID (14). However, the link between inhibition of HDAC and apoptosis remains elusive.

Here we investigated three HDIs: FR901228, TSA, and butyrate. Induction of p21 was caused by all three inhibitors and did not determine their cytotoxicity. The cytotoxicity was correlated with mitotic arrest. Taking into account rapid cell death after exposure to FR901228 or TSA, we suggest that a nontranscriptional mechanism may be involved in mitotic arrest and apoptosis. We found that TSA caused dramatic acetylation of tubulin and microtubules, which correlated with apoptosis. This suggests that nontranscriptional effects of HDIs such as acetylation of proteins, which are differentially affected by HDIs, may, in part, be responsible for differential cytotoxicity of HDIs.

Materials and Methods

Cell Lines and Reagents. HL60 and Jurkat, human leukemia cell lines, A549, a human lung cancer cell line, and MCF-7, a breast cancer cell line, were obtained from American Type Culture Collection (Manassas, VA). PTX (Taxol), was a Bristol-Myers product (Bristol-Myers, Princeton, NJ). TSA was obtained from Wako Pure Chemical Industries, Ltd. and was prepared as 1 mg/ml stock in DMSO. Sodium butyrate (Bu) was obtained from Sigma. FR901228 (depsipeptide) was obtained from Chemistry and Synthesis Branch (National Cancer Institute, Bethesda, MD) and prepared as a 1-mg/ml stock solution in water.

Immunoblot Analysis. Proteins were resolved with SDS-PAGE (15) or NuPAGE 4–12% Bis-Tris gel with 4-morpholinepropanesulfonic acid (MOPS) running buffer (NOVEX, San Diego, CA) according to the manufacturer's instructions. Immunoblotting was performed using rabbit polyclonal antihuman PARP (Upstate Biotechnology, Lake Placid, NY), mouse monoclonal antihuman WAF1 (EA10; Oncogene Res., Calbiochem), rabbit polyclonal antiacetylated histone H3 (Upstate Biotechnology), mouse monoclonal antihuman tubulin and anti-Lys40-acetotubulin, both obtained from Sigma (St. Louis, MO).

MTT Assay. Fifteen thousand floating (HL60, Jurkat) cells or 2,000 MCF-7, A549 cells were plated in 96-well flat-bottomed plates and then exposed to tested agents. After 3 days, 20 μl of 5 mg/ml MTT solution in PBS was added to each well for 4 h. After removal of the medium, 170 μl of

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[2] The abbreviations used are: HDAC, histone deacetylase; PARP, poly-(ADP-ribose) polymerase; HDI, HDAC inhibitor; TSA, trichostatin A; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole; PTX, paclitaxel.

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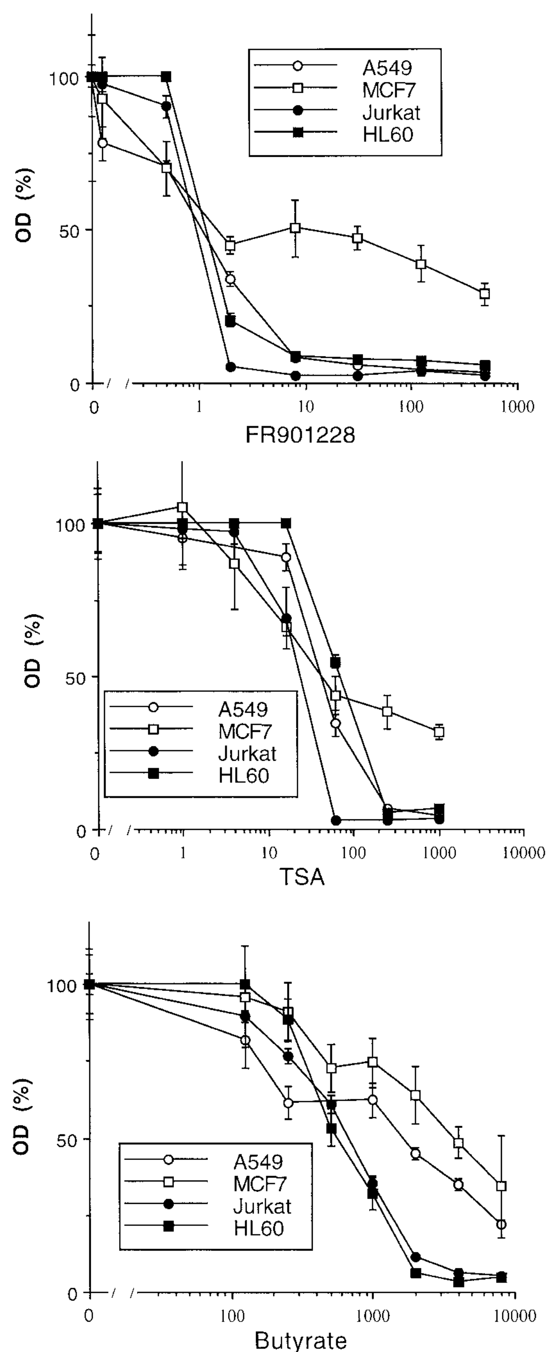

Fig. 1. Comparison of different cell lines. As indicated, A549, MCF-7, Jurkat, and HL60 cells were incubated with FR901228 (ng/ml), TSA (ng/ml), or butyrate (μM). MTT assay was performed after 3 days as described in "Materials and Methods." Results were calculated as the percentage of values obtained with untreated cells and represent mean ± SD.

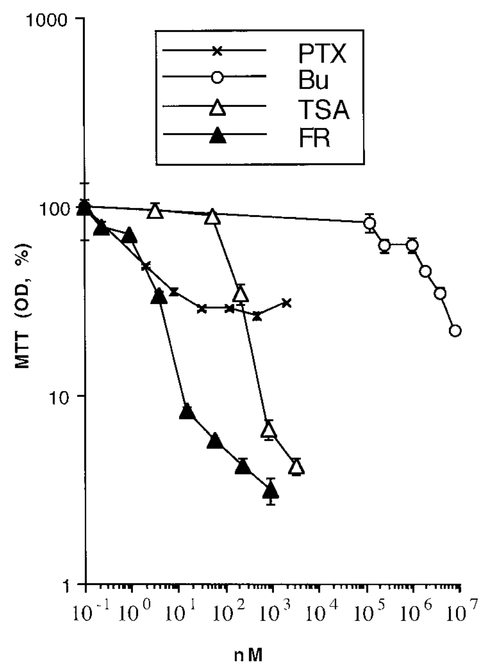

Fig. 2. Potencies and maximal cytotoxicities of HDIs. A549 cells were incubated with PTX, FR901228 (*FR*), TSA, or butyrate (*Bu*). MTT assay was performed after 3 days as described in "Materials and Methods." Results were calculated as the percentage of values obtained with untreated cells and represent mean ± SD.

DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined (15). Triplicate wells were assayed for each condition, and SDs were determined.

Number of Dead and Live Cells. Cells were plated in 24-well plates in 1 ml of medium, or in 96-well plates in 0.2 ml, and were treated with drugs. After the indicated time, cells were counted in triplicate on a Coulter Z1 cell counter (Hialeah, FL). In addition, cells were incubated with trypan blue, and the numbers of blue (dead) cells and transparent (live) cells were counted in a hemocytometer.

Cell Cycle Analysis. Cells were incubated for 30 min in propidium iodide staining solution containing 0.05 mg/ml propidium iodide (Sigma), 1 mM EDTA, 0.1% Triton X-100, and 1 mg/ml RNase A in PBS. The suspension was then passed through a nylon mesh filter and analyzed on a Becton Dickinson FACScan.

Mitotic Index. Cells were stained with DAPI as described previously. Nuclei were visualized by UV microscopy (16).

Results

Cytotoxicity and G_2-M Arrest Caused by HDIs. We first compared the cytotoxicity of HDIs (FR901228, TSA, and butyrate) in a panel of human cell lines including cell lines that easily undergo apoptosis (Jurkat and HL60) and those that are resistant to PTX-induced apoptosis (MCF-7 and A549) cell lines (15–17). Jurkat and HL60 cells were sensitive to all of the HDIs. In these leukemia cells, MTT values dropped to background (Fig. 1). MCF-7 cells which lack caspase-3, were relatively resistant to all of the HDIs. In A549, MTT values were not completely inhibited at any concentration of butyrate after 3 days of treatment (Fig. 1). Unlike butyrate, both FR901228 and TSA quantitatively killed A549 cells (Figs. 1 and 2). For this cell line, the cytotoxic effects of all three HDIs are shown in Fig. 2. As determined by IC50, FR901228 was

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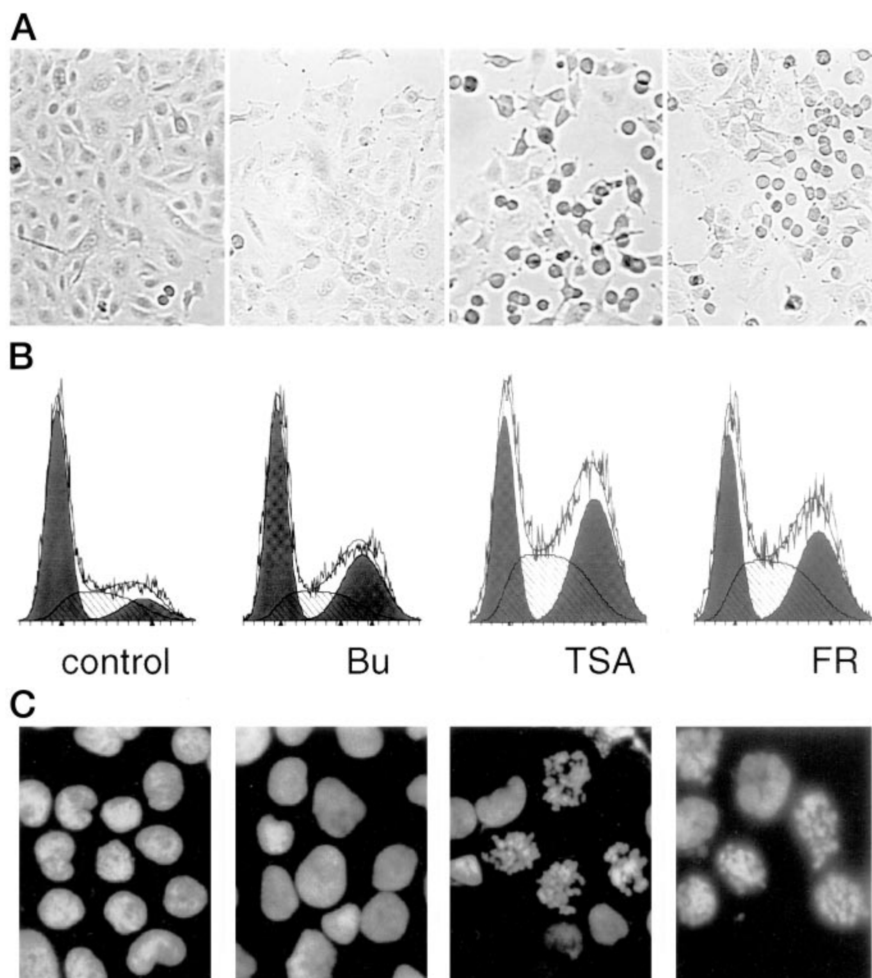

Fig. 3. G_2-M arrest caused by HDIs. In *A*, A549 cells were incubated with 5 mM butyrate (*Bu*), 100 ng/ml (300 nM) TSA, or 10 ng/ml (20 nM) FR901228 or left untreated (*control*). After 16 h, photomicroscopy of live culture was performed. *B*, cells treated as indicated, and flow cytometry was performed after 16 h as described in "Materials and Methods." C, cells treated as indicated, and DAPI staining was performed, as described in "Materials and Methods."

100 times more potent than TSA and 1,000,000 times more potent than butyrate. In addition, FR901228 and TSA were more cytotoxic than butyrate. This indicates that, in comparison with TSA and FR901228, butyrate lacks certain cytotoxic activities, at least in achievable concentrations (5–10 mM).

Recently it has been shown that FR901228 causes mitotic arrest (18), and that the treatment with HDIs causes defects in chromosome segregation in mitosis (19). Here we compared FR901228 with other HDIs, *i.e.,* TSA and butyrate. Cell cycle analysis showed that all three HDIs caused G_1 and G_2-M arrest of A549 (Fig. 3). However, butyrate predominantly caused G_2 arrest, whereas TSA and FR901228 caused both G_2 and mitotic arrest. As shown in Fig. 2, treatment of A549 cells with TSA or FR901228 (but not with butyrate) caused rapid rounding of 15–20% cells. This appearance is a characteristic of the cell culture treated with TSA or FR901228 (Fig. 3*A*). By DAPI staining, all of the round cells were arrested at prometaphase of mitosis (Fig. 3*C*). Unlike TSA and FR901228, butyrate did not increase the number of mitotic cells.

Effects of HDIs and PTX on p21, Tubulin Acetylation, and Polymerization. As we demonstrated, TSA and FR901228 were more toxic than butyrate in A549 cells. Nevertheless, TSA, FR901228, and butyrate all caused comparable p21 induction (Fig. 4). Because both TSA and FR901228 caused mitotic arrest, we next investigated their effects on tubulin polymerization. Soluble and insoluble tubulin were separated as described previously (16). As shown in Fig. 4, most tubulin was soluble in untreated cells under these assay conditions. PTX stabilized tubulin polymers, as evidenced by a decrease of soluble tubulin and an increase of insoluble tubulin. Unlike PTX, none of the HDIs affected the soluble:insoluble tubulin ratio. Next, we investigated modifications of tubulin. Inhibitors of HDACs in theory may inhibit putative tubulin deacetylases. Using commercially available antibodies, we found that TSA induced an increase in tubulin acetylation on lysine 40 of α-tubulin (Fig. 4). This acetylation occurred in both soluble and insoluble tubulin and did not affect its polymerization.

Tubulin Acetylation and Apoptosis. Among the four cell lines evaluated, Jurkat cell line was the most sensitive to the cytotoxic effect of TSA. We next investigate the association between tubulin acetylation and apoptosis. Maximal acetylation of tubulin was achieved at 50 ng/ml TSA. This was accompanied by PARP cleavage, a marker of caspase activation. Similarly, maximal cytotoxicity was already detectable at 50 ng/ml TSA (Fig. 5). In addition to inducing a G_2-M

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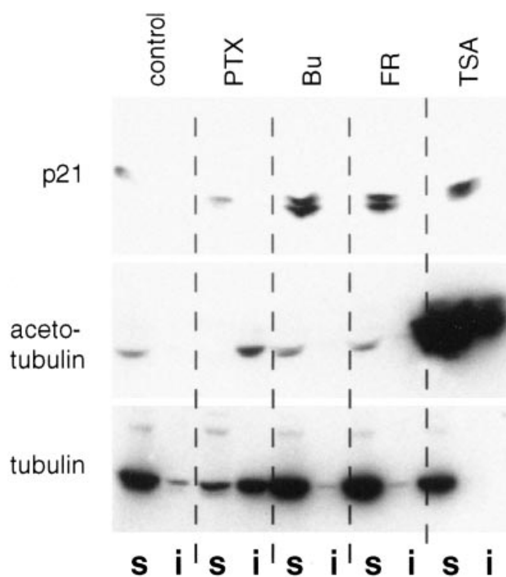

Fig. 4. Effects of HDI and PTX on p21, tubulin polimerization and acetylation. A549 cells were incubated with indicated drugs [100 ng/ml PTX; 5 mM (550,000 ng/ml) Bu; 10 ng/ml FR901228; and 100 ng/ml TSA] for 16 h, and then were lysed and NP40-soluble (*s*) and insoluble (*i*) proteins were then solubilized in SDS loading buffer. Immunoblots for p21, acetylated tubulin, and tubulin were performed.

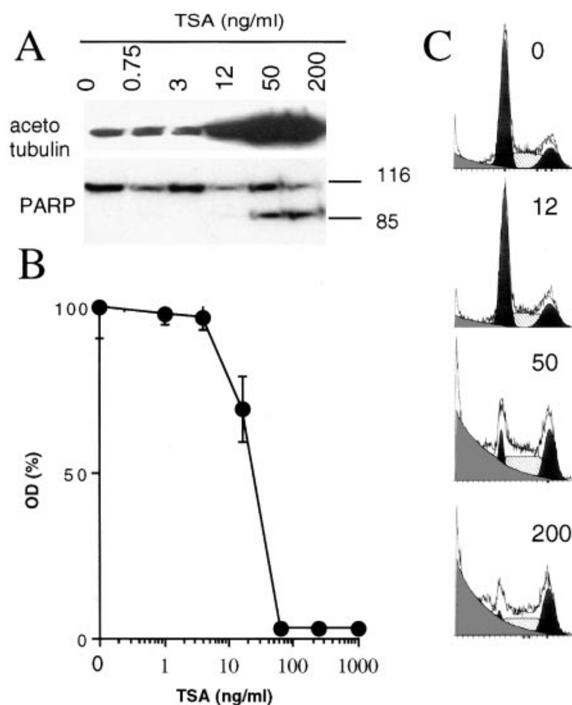

Fig. 5. Dose response of acetylation of tubulin and cell death. Jurkat cells were treated with the indicated concentrations of TSA. In *A*, after 16 h, immunoblot assay for acetylated tubulin and for PARP was performed as described in "Materials and Methods." In *B*, MTT assay was performed after 3 days, as described in "Materials and Methods." Results were calculated as the percentage of values obtained with untreated cells and represent mean ± SD. In *C*, cells were treated with indicated concentrations of TSA, and flow cytometry was performed after 20 h.

arrest, 50 ng/ml TSA caused the appearance of a sub-G_1 peak, a marker of apoptosis. Importantly, the acetylation of tubulin was detectable at 12 ng/ml TSA, a concentration that was only marginally cytotoxic. Thus, tubulin acetylation parallels apoptosis.

Acetylation of Tubulin Is an Early Event. We next compared the time course of p21 induction and tubulin acetylation. Induction of p21 was detected by 4 h with maximal induction by 8 h (Fig. 6). Acetylation of tubulin occurs rapidly after the addition of TSA. Maximal acetylation of tubulin was evident by 1 h (Fig. 6). Such a rapid tubulin acetylation preceded any signs of apoptosis.

Discussion

Most known effects of HDIs are mediated by alterations of gene expression, including *p21*. In the cells studied here, FR901228, TSA, and butyrate caused comparable *p21* induction. It has been reported that cells that are resistant to butyrate-induced apoptosis show markedly enhanced *p21* expression (20). In a cell type-dependent manner, p21 is required for G_1 arrest but not for cell death (11). Interestingly, arsenic causes mitotic arrest leading to apoptosis (21, 22). Even more intriguingly, arsenic acetylates histones (23). Here we showed that HDIs varied in their abilities to cause mitotic arrest and cell death. Although the acetylation of histones causes p21 induction and G_1 arrest, G_2-M arrest and cell death may result from the inhibition of other targets. We have identified a non-histone target that appears to be involved in cell death caused by TSA. Acetylation of lysine 40 of α-tubulin occurs early after the addition of TSA and correlates with the induction of apoptosis. It has been shown that acetylation of tubulin does not affect its temperature-dependent polymerization or depolymerization *in vitro* (24). Here we extended those findings to show that acetylation of tubulin did not affect polymerization in intact cells. Given that FR901228 does not cause an increase in tubulin acetylation, we conclude that it is dispensable for both mitotic arrest and apoptosis caused by FR901228. Yet, this finding demon-

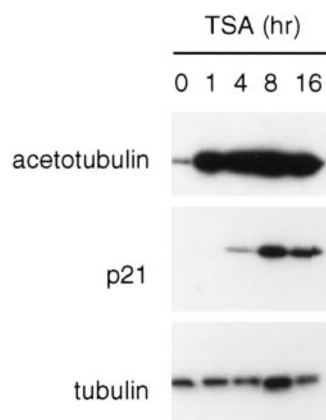

Fig. 6. Time response of acetylation of tubulin and p21 induction. Jurkat cells were incubated with 100 ng/ml TSA for indicated time. Immunoblots for p21, acetotubulin, and tubulin were performed as described in "Materials and Methods."

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strates the existence of cytoplasmic targets that are differentially affected by HDIs. While our article was under review, it has been reported that TSA inhibits HDAC6, a microtubule-associated deacetylase, and causes acetylation of lysine 40 of α-tubulin (25). Unlike other family members, HDAC6 is uniquely resistant to trapoxin-B and sodium butyrate. These data explain the acetylation of tubulin by TSA but not by FR901228 and TSA observed in our study.

Putative target(s) responsible for the initiation of apoptosis would be sensitive to FR901228 and TSA but less sensitive to butyrate. If one considers that the most effective anticancer agents have multiple mechanisms of action, or multiple events emanating from a single activity, then the identification of mechanisms of cell death beyond altered gene expression becomes very relevant in predicting the eventual importance of HDIs in anticancer therapy. The identification of proteins, the acetylation of which leads to cell death, remains a major challenge.

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