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Parthenolide Specifically Depletes Histone Deacetylase 1 Protein and Induces Cell Death through Ataxia Telangiectasia Mutated

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SUMMARY

Histone deacetylases (HDACs), enzymes involved in chromatin remodeling, are promising targets for anticancer drug development. Several HDAC inhibitors (HDACi) are in clinical trials. One limitation of present HDACi is their nonspecificity, affecting many HDACs with similar effectiveness. We have identified a small molecule, the sesquiterpene lactone parthenolide (PN), which specifically depletes HDAC1 protein without affecting other class I/II HDACs. HDAC1 depletion occurred through proteasomal degradation and resulted in transcriptional consequences comparable to those observed with pan-HDACi. Surprisingly, HDAC1 depletion did not occur through the inflammation mediator IKK2, a known PN target and regulator of HDAC1. Rather, PN promoted HDAC1 depletion and cell death through the DNA-damage-transducer ataxia telangiectasia mutated. Our study suggests that modulating cellular HDAC protein levels with small molecules provides an alternative approach to specific HDAC inhibition and effective cancer treatment.

INTRODUCTION

Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from acetyllysine residues in proteins. Researchers have identified 18 human HDACs that are grouped into four classes based on their sequence homology [1, 2]. HDACs were originally identified as epigenetic regulators of gene expression that deacetylate histone H3 and histone H4 N-terminal tails and promote transcriptionally silent chromatin [3]. However, HDACs also catalyze the deacetylation of many nonhistone proteins like p53, signal transducer and activator of transcription 3, nuclear factor-κB (NF-κB), Bax, and hypoxia-inducible factor 1, thereby regulating their activities through changes in their stability, protein-protein interactions, and subcellular localization [4, 5].

Studies over the past decade have consistently indicated that several HDACs play important roles in promoting oncogenesis. Foremost is HDAC1, which is overexpressed in several cancers and is a critical transcriptional repressor of the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, which normally suppress cell proliferation [6, 7]. This has spurred the development of several HDAC inhibitors (HDACi), some of which have shown impressive pharmacological properties and are in the final stages of clinical development [4, 8]. However, most all HDACi are pan-HDAC inhibitors, enzymatically inhibiting class I, II, and IV HDACs with comparable efficiency. The inhibition of multiple HDACs can have undesirable consequences; thus the search for more specific HDACi remains an ongoing pursuit.

The activity of HDACs is regulated at many levels [9]. For example, the class I HDACs HDAC1, HDAC2, and HDAC3 are known to be associated with multiprotein complexes Sin3, NuRD, and SMRT/N-CoR, and it is through these complexes that HDACs are both enzymatically active and functionally competent to recognize their appropriate substrates [10–12]. HDAC activity can also be regulated both positively and negatively by post-translational modifications, including phosphorylation and sumoylation. These modifications can affect HDAC association with multiprotein complexes, as is the case with HDAC1 C-terminal phosphorylation and its association with the Sin3 complex, or the subcellular localization of HDAC proteins, as is the case with N-terminal phosphorylation of class II HDACs 4, 5, 7, and 9 and their cytoplasmic localization promoted by 14-3-3 binding [13, 14]. HDACs can be transcriptionally autoregulatory (e.g., HDAC1) or cross-regulatory (e.g., HDAC1 affecting HDACs 2 and 3), and splice variants exist for class II HDACs 3 and 9 and the class IV HDAC11 [7, 15–17]. In addition, all class III HDACs, otherwise known as sirtuins, are completely dependent on the cofactor NAD^+, and regulating NAD^+ levels or the levels of competitive inhibitors NADH and nicotinamide can affect class III HDAC function [18].

Previously, we had found that the proinflammatory agents such as the cytokine tumor necrosis factor-α (TNF-α) induced ubiquitination-mediated proteasomal degradation of HDAC1 through activated IκB kinase 2 (IKK2) [19]. HDAC1 depletion by TNF-α caused a decrease in HDACs 1 and 2 associated with the p21 promoter, increased local and global histone H3 acetylation, and

upregulation of p21 mRNA and protein, much as would occur following treatment with pan-HDACi. Notably, the depletion caused by TNF-α treatment was specific for HDAC1 and speaks to the importance of this HDAC in regulating the expression of many genes.

While investigating small-molecule inhibitors of TNFα-mediated HDAC1 depletion, we unexpectedly discovered that the IKK2 inhibitor parthenolide (PN) (Figure 1) itself did not inhibit but, instead, promoted HDAC1 depletion. PN is the primary bioactive agent in feverfew, a traditional herbal medicine used for the treatment of fever, migraines, arthritis, and superficial inflammation [20]. Structure-function relationship studies have indicated that the epoxide and α-methylene-γ-lactone moieties of this sesquiterpene lactone compound are responsible for its activity, which allows PN to readily react with nucleophilic sites on biological molecules such as exposed cysteines, thereby inactivating critical proteins [21]. Mechanistically, the anti-inflammatory effects of PN are thought to stem from its inhibition of canonical $p65^{RELA}$/p50 NF-κB activation by the IKK signalsome, through direct interaction of PN with IKK2 [22, 23]. However, PN has also been shown to promote apoptosis of cancer cells through additional mechanisms, including increasing intracellular concentrations of reactive oxygen species (ROS) and sustained activation of the c-Jun N-terminal kinase (JNK) [24–26]. In addition, while its exact mechanism is unknown, PN is the only reported small molecule demonstrating selective toxicity against cancer stem cells [27]. Here, we report that PN specifically depletes HDAC1 through ubiquitination and proteasomal degradation by activating the signal transducer protein ataxia telangiectasia mutated (ATM) independent of IKK2 and JNK1/2.

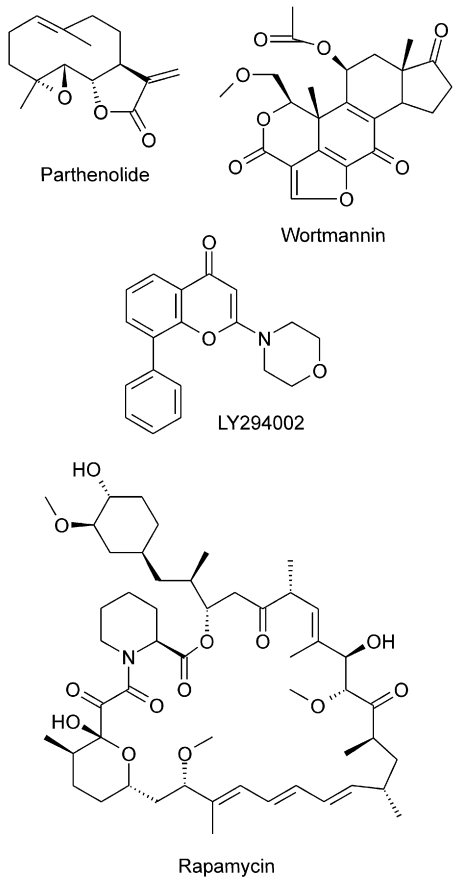

Figure 1. Chemical Structures of Parthenolide, PN, and Kinase Inhibitors Used in This Study

RESULTS

PN Specifically Depletes HDAC1 through Proteasomal Degradation

PN is a known inhibitor of IKK2 [23], and IKK2 activity is required for TNFα-mediated HDAC1 depletion [19]. Thus, it was quite unexpected when we discovered that PN treatment alone actually depleted HDAC1 protein from ZR-75-1 human breast carcinoma cells in a time- and concentration-dependent manner (Figures 2A and 2B). We observed a similar HDAC1 depletion in many PN-treated cancer cells and transformed cells of different lineages (see Figure S1 in the Supplemental Data available with this article online). This depletion was specific for HDAC1 as we found no observable changes in the protein levels or cytoplasmic/nuclear distribution of the other class I and class II HDAC proteins tested (Figures 2A and 2B). HDAC1 depletion was also reversible, with HDAC1 levels returning to normal 24 hr after PN treatment (Figure 2C). It should be noted that these changes in HDAC1 protein levels occurred with PN concentrations and cell exposure times comparable to those routinely used to explore the biological effects of PN [21–26].

HDAC1 is primarily a nuclear protein [2]. However, its depletion following PN treatment did not result from a change in its subcellular localization (Figures 2A and 2B), nor from its sequestration into an insoluble fraction (Figure 2D). A real-time quantitative reverse transcription (RT)-polymerase chain reaction (PCR) assay did not reveal any reductions in HDAC1 mRNA levels (Figure 2E), suggesting that changes in transcription of the *HDAC1* gene or stability of HDAC1 mRNA were not responsible for PN-mediated HDAC1 depletion. Similarly, HDAC1 depletion was likely not the result of PN-mediated inhibition of HDAC1 deacetylase activity, because loss of HDAC1 activity correlated directly with loss of HDAC1 protein (Figure 2F). In fact, we observed no inhibition of deacetylase activity following direct PN treatment of immunoprecipitated HDAC1 (Figure 2F). However, treatment with the proteasomal inhibitor MG-132 but not the pan-caspase inhibitor Z-VAD-FMK blocked the depletion of HDAC1 (Figure 2G), suggesting that ubiquitination and proteasomal degradation are responsible for PN-mediated HDAC1 depletion. This was confirmed through immunoprecipitation experiments, where MG-132-treated cells were found to contain greatly increased quantities of slower mobility, ubiquitinated HDAC1 protein (Figure 2H). In addition, previous studies have found that lysine residues within the C-terminal region of HDAC1 (amino acids 432–482) may be

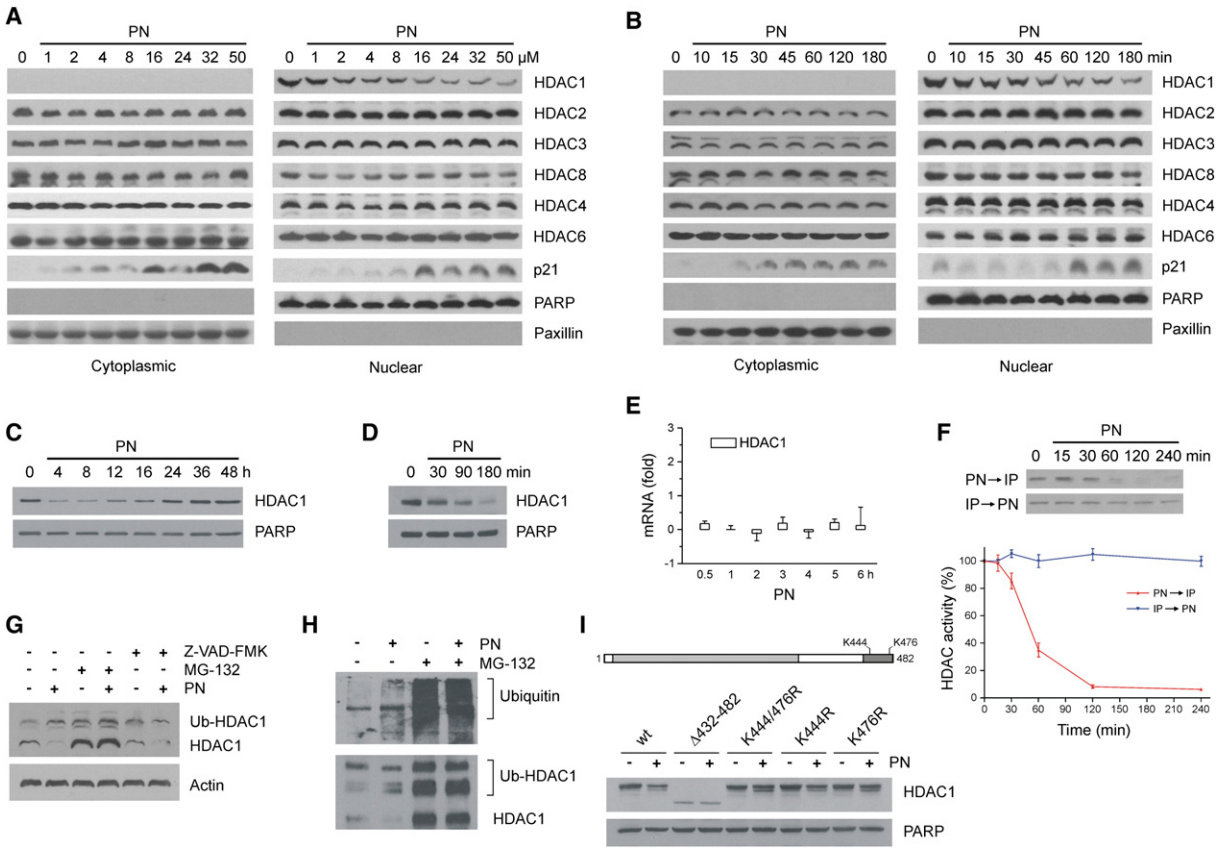

Figure 2. PN Specifically Depletes HDAC1 Protein through Ubiquitination and Proteasomal Degradation

(A) Western blots of cytoplasmic and nuclear extract proteins from ZR-75-1 cells treated with PN at increasing concentrations for 3 hr and probed with the indicated antibodies (right). Paxillin and poly(ADP-ribose) polymerase (PARP) blots served as protein-loading controls and as controls for cytoplasmic and nuclear extract integrity.

(B) Western blots performed as described in (A) but with 15 μM PN and increasing treatment durations.

(C) Western blots of nuclear extract proteins from ZR-75-1 cells treated with 15 μM PN for extended durations.

(D) Western blots of Laemmli loading buffer-extractable proteins from ZR-75-1 cells treated with 15 μM PN for the times indicated.

(E) HDAC1 mRNA levels in ZR-75-1 cells treated with 15 μM PN as determined using a real-time quantitative RT-PCR assay after normalization against glyceraldehyde-3-phosphate dehydrogenase mRNA levels. Error bars indicate standard error of the mean.

(F) Western blots of HDAC1 protein immunoprecipitated with anti-HDAC1 antibodies from ZR-75-1 cells treated with 15 μM PN for increasing durations (PN→IP) or immunoprecipitated from untreated ZR-75-1 cells and then treated with 15 μM PN for increasing durations in vitro (IP→PN) (top panel). Histone deacetylase activity measurements of HDAC-1 protein immunoprecipitated from PN-treated ZR-75-1 cells (PN→IP, red triangles) or immunoprecipitated HDAC1 from untreated ZR-75-1 cells subsequently treated with PN in vitro (IP→PN, inverted blue triangles) (bottom panel). Error bars indicate standard error of the mean.

(G) Western blots of whole cell extract proteins from ZR-75-1 cells treated for 3 hr with 10 μM MG-132, 10 μM Z-VAD-FMK, and 15 μM PN as indicated.

(H) Western blots of HDAC1 protein immunoprecipitated from ZR-75-1 cells treated with 15 μM PN and/or 10 μM MG-132 for 3 hr, as indicated, and probed with ubiquitin antibodies (top panel) or stripped and reprobed with HDAC1 antibodies (bottom panel).

(I) Schematic representation of HDAC1 protein illustrating its histone deacetylase domain (amino acids 10–321, light gray) and lysine-rich carboxy terminus (amino acids 432–482, dark gray) (top panel). Western blots of nuclear extract proteins from 293T cells expressing the C-terminal FLAG epitope-tagged mutant HDAC1 proteins indicated following treatment of these cells with or without 15 μM PN for 3 hr as indicated.

post-translationally modified with ubiquitin and SUMO-1 (small *u*biquitin-related *mo*difier) [28]. Using plasmid vectors expressing HDAC1 mutant proteins and transient transfections, we found that this same region is also required for PN-mediated HDAC1 depletion; with intermediate effects being observed when both critical residues K444 and K476 are mutated and lesser effects being observed when only one site is mutated (Figure 2I). Note that the HDAC1 Δ432–482 protein is less abundant than the other HDAC1 proteins. This is understandable, given that it is a predominantly cytoplasmic protein [28]. Taken together, these data suggest that PN specifically depletes HDAC1 protein through ubiquitination and proteasomal degradation, much as was previously found for HDAC1 depletion following TNF-α treatment [19].

HDAC1 Depletion by PN Leads to p53-Independent p21 Upregulation and Global Histone Acetylation

The *CDKN1A* gene, which encodes the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, is one of the few genes that

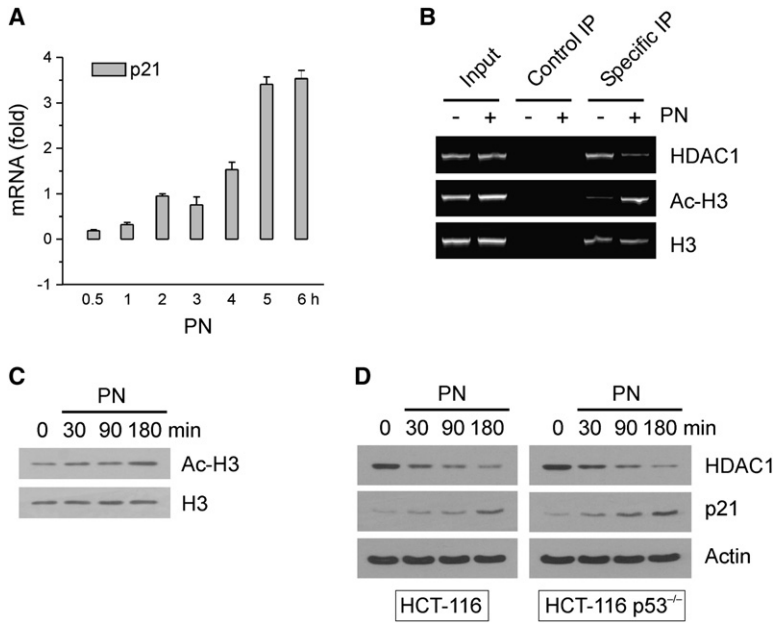

Figure 3. PN-Mediated HDAC1 Depletion Upregulates p21$^{WAF1/CIP1}$ and Acetylated Histone H3 Levels through p53-Independent Mechanisms

(A) Quantitative RT-PCR analysis of p21 mRNA levels in ZR-75-1 cells treated with 15 μM PN. Error bars indicate standard error of the mean.

(B) Ethidium bromide-stained PCR products obtained from ZR-75-1 cells treated with 15 μM PN for 3 hr as indicated, processed by using the chromatin immunoprecipitation (ChIP) assay with either the indicated antibodies (right) or nonimmunogenic control antibodies and PCR amplified with primers specific for the p21 proximal promoter. IP, immunoprecipitation; Ac-H3, acetylated histone H3.

(C) Western blots of nuclear extract proteins from ZR-75-1 cells treated with 15 μM PN and probed with antibodies against total and acetylated histone H3.

(D) Western blots of whole-cell extract proteins from normal and p53$^{-/-}$ HCT-116 cells treated with 15 μM PN.

are consistently upregulated after cell exposure to pan-HDAC inhibitors [29]. Similar results have been observed with cells treated with siRNA against HDAC1 [30]. We also observed a considerable increase in p21 protein after PN treatment, which coincided with the disappearance of HDAC1 (Figures 2A and 2B). This upregulation could be explained by the substantial increase in p21 mRNA observed after PN treatment, as determined by using real-time RT-PCR (Figure 3A). This increase in p21 mRNA most likely resulted from the loss of HDAC1 associated with the *CDKN1A* promoter, increased local histone H3 acetylation, and increased transcription of the *CDKN1A* gene, as shown in our chromatin immunoprecipitation (ChIP) assay data (Figure 3B).

Another expected consequence of pan-HDAC inhibition is an increase in cellular acetylated histone H3 levels [31]. We also observed this after PN treatment of ZR-75-1 cells (Figure 3C), though not to the same degree as observed after TNFα treatment [19]. This difference in histone acetylation could reflect differences in global histone acetyltransferase activity following each treatment. However, our findings suggest that specific HDAC1 depletion by either approach yields a global increase in acetylated histone H3 levels.

It is well known that the *CDKN1A* gene is under the control of the p53 transcription factor and that the ZR-75-1 cells contain wild-type p53 [32]. To test whether p53 is involved with the PN-dependent upregulation of p21, we tested HDAC1 and p21 responses to PN treatment in matched p53 wild-type (HCT-116) and deficient (HCT-116 p53$^{-/-}$) cells. We found that PN treatment depleted HDAC1 protein and caused upregulation of p21 in both cell lines (Figure 3D). We interpreted these results as indicating that p53 is unnecessary for the specific depletion of HDAC1 by PN and that the upregulation of p21 by PN does not depend on p53.

HDAC1 Depletion by PN Does Not Require Signal Transduction through IKK2

PN is known to inhibit IKK2 kinase activity and thereby inhibit signaling through NF-κB [21–23]. We have also found that IKK2 plays a key role in TNFα-dependent HDAC1 depletion [19]. Using wild-type mouse embryo fibroblasts (MEFs) and MEFs containing homozygous deletions in IKK2, RelA, and IκBα, we found no evidence that the absence of these proteins had any effect on HDAC1 depletion following PN treatment (Figure 4A). These data would strongly suggest that the IKK→IκBα→NF-κB signal transduction pathway is not required for the depletion of HDAC1 by PN. Likewise, although PN has been shown to stimulate JNK activity [26], absence of JNK1 or JNK2 had no effect on PN-mediated HDAC1 depletion (Figure 4B). Thus, there appears to be no absolute requirement for both JNK proteins for the depletion of HDAC1 protein by PN.

The Phosphoinositide-3-Kinase-like Kinase ATM Is Required for PN-Mediated HDAC1 Depletion

To elucidate possible signal transduction pathways involved in PN-mediated HDAC1 depletion, cells were treated with different kinase inhibitors along with PN. Low concentrations of the kinase inhibitor wortmannin (Figure 1), which inhibits phosphoinositide-3-kinase (PI3K), had little effect on PN-mediated HDAC1 depletion and subsequent p21 upregulation (Figure 5A). However, high concentrations of wortmannin, which can inhibit several phosphoinositide-3-kinase-like kinases (PIKKs) [33], significantly inhibited PN-mediated depletion of HDAC1 as well as p21 protein accumulation (Figure 5B). In addition, the more specific PI3K inhibitor LY294002 (Figure 1) and the mammalian Target of Rapamycin (mTOR) inhibitor rapamycin (Figure 1) had no similar effects on HDAC1 and p21 (Figure 5B). These data suggested that another PIKK may be involved in these PN-mediated effects.

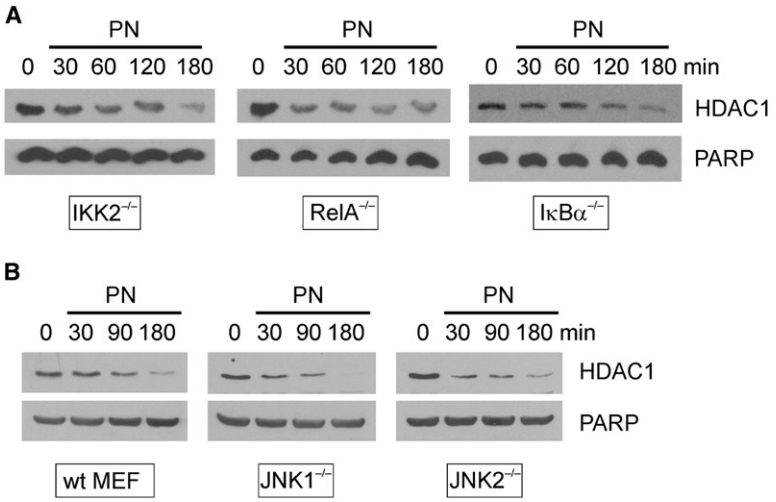

Figure 4. HDAC1 Depletion Induced by Treatment with PN Does Not Require Intact IKK→IκBα→NF-κB or JNK Signal-Transduction Pathways

(A) Western blots of nuclear extract proteins from MEFs with homozygous deletion of IKK2, RelA, or IκBα genes treated with 15 μM PN for the indicated times and probed with antibodies against HDAC1 and poly(ADP-ribose) polymerase (PARP) as indicated (right).

(B) Western blots performed as described in (A) but with wild-type (wt) and JNK1$^{-/-}$ and JNK2$^{-/-}$ MEFs.

Three PIKKs-ATM, ataxia-telangiectasia- and Rad3-related kinase (ATR), and DNA-dependent protein kinase (DNA-PK) transduce DNA-damage signals to activate p53 [34–36]. Cells deficient in functional ATR and DNA-PK did not significantly affect HDAC1 depletion (Figures 5C and 5D). However, cells containing a homozygous deletion of ATM (ATM$^{-/-}$) showed significantly reduced levels of HDAC1 depletion following treatment with PN (Figure 5E). Reduced HDAC1 depletion and p21 upregulation after PN treatment could also be observed in a cell line containing wild-type ATM (ZR-75-1), after treatment with ATM siRNA (Figure 5F). These data strongly suggest that ATM is involved in PN-mediated HDAC1 depletion and its downstream effects.

PN-Mediated Cell Death Occurs through ATM

PN is known to induce apoptosis in many human cancer cell lines [37]. Several different mechanisms have been proposed, including (1) redox disruption, (2) endoplasmic reticulum stress, (3) caspase 8 activation, (4) changes in proapoptotic Bcl-2 proteins, (5) suppression of NF-κB signaling, and (6) sustained JNK activation. However, the exact contributions of these mechanisms and the pathways involved remain obscure. To test the involvement of the NF-κB, JNK, and DNA damage-related PIKK signaling pathways, cell lines deficient in these different activities were subjected to PN treatment for 48 hr prior to assaying their metabolic activity by using the WST-1 cell-proliferation reagent. MEFs deficient in IKK2, RelA, or IκBα had essentially the same response to PN as their wild-type counterpart (Figure 6A). Thus, the IKK→IκBα→NF-κB signal transduction pathway is not required for PN's effects on proliferation and viability of these cells. Nearly equivalent effects on viability were observed with wild-type, JNK2$^{-/-}$, and JNK1$^{-/-}$ MEFs following PN treatment (Figure 6B). Thus, the JNK proteins may also not play a major role in promoting PN-mediated cell death.

With regards to the PIKKs and PN-mediated cell death, suppression of ATR activity through the inducible expression of a dominant-negative mutant protein resulted in slightly increased PN sensitivity, especially at low concentrations (Figure 6C). These data suggest that wild-type ATR protein is not required for PN's cytotoxicity; rather, ATR may actually be involved in a process that reduces PN's cytotoxic effects. DNA-PK, on the other hand, apparently plays no significant role in either process because no apparent differences were found in the viability of wild-type and DNA-PK$^{-/-}$ cells following PN treatment (Figure 6D). However, a striking difference in viability was observed between ATM-containing and ATM-deficient cells following PN treatment, with ATM$^{-/-}$ cells exhibiting no detectable sensitivity to PN within the concentration range tested (Figure 6E). These data would strongly suggest that PN's effects on cell proliferation and viability absolutely require ATM.

One of the prime downstream targets of ATM is p53, whose activation leads to either cell-cycle arrest or apoptosis through multiple mechanisms [32, 34]. Thus, we investigated whether p53 is involved in PN-mediated cell death. MEFs deficient in p53 exhibited only slightly less sensitivity to PN than their wild-type counterparts at intermediate PN concentrations (Figure 6F). Thus, these data would suggest that p53 is not the sole mediator of PN cytotoxicity and that other proteins downstream of ATM may be involved.

DISCUSSION

Previously, we had found that activation of IKK2 by proinflammatory agents caused a specific cellular depletion of HDAC1 protein levels [19, 38]. Contrary to our expectations, however, we found in our present studies that the IKK2-specific inhibitor PN itself promoted HDAC1 depletion. As with treatment with proinflammatory agents such as TNF-α and interferon-γ, the effect of PN was specific for HDAC1, with the cellular levels and subcellular distribution of other class I and class II HDACs being unaffected. HDAC1 depletion was observed in many cancer cell types, as was also previously observed. Likewise, HDAC1 depletion occurred through ubiquitination and

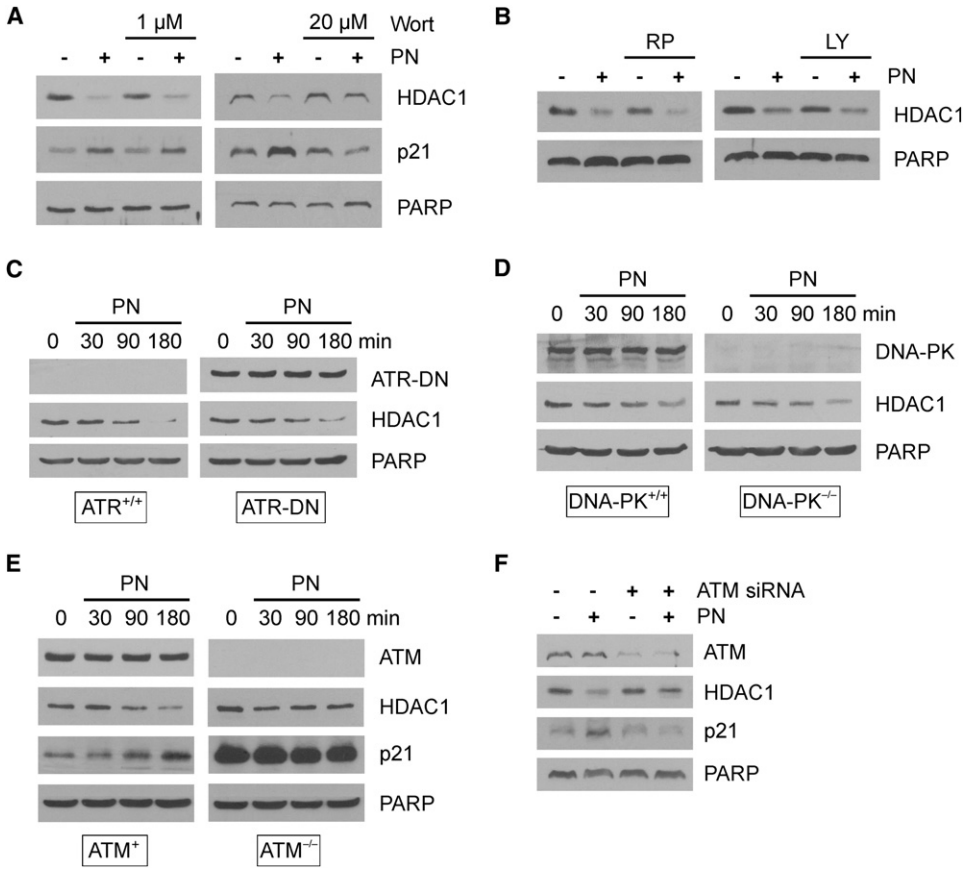

Figure 5. PN-Mediated HDAC1 Depletion Requires the PIKK ATM

(A) Western blots of nuclear extract proteins from ZR-75-1 cells treated with 15 μM PN and 1 μM or 20 μM wortmannin (Wort) for 3 hr as indicated.

(B) Western blots of nuclear extract proteins from ZR-75-1 cells treated with 15 μM PN and 1 μM rapamycin (RP) or 10 μM LY294002 (LY) for 3 hr as indicated.

(C) Western blots of nuclear extract proteins from uninduced GK41-U2OS cells (left panel) containing wild-type ATR ($ATR^{+/+}$) or doxycycline-induced GK41-U2OS cells expressing (right panel) a dominant negative mutant of the PIKK ATR (ATR-DN) treated with 15 μM PN.

(D) Western blots of nuclear extract proteins from cells possessing (M059K, left panel) or lacking (M059J, right panel) the PIKK DNA-PK treated with 15 μM PN.

(E) Western blots of nuclear extract proteins from cells possessing (AT22IJE-TpEBS7-YZ5, left panel) or lacking (AT22IJE, right panel) the PIKK ATM treated with 15 μM PN.

(F) Western blots of nuclear extract proteins from ZR-75-1 cells treated with ATM siRNA for 72 hr and 15 μM parthenolide for the final 3 hr, as indicated.

proteasomal degradation, and the consequences of PN-mediated HDAC1 depletion (e.g., loss of HDAC1 associated with the p21 promoter, increased local and global histone H3 acetylation, and upregulation of p21 mRNA and protein) mirrored those observed following TNF-α treatment. Additionally, as previously seen, these effects were not dependent on p53. However, unlike our previous studies with proinflammatory agents, PN-mediated HDAC1 depletion was unaffected by the absence of IKK2. Thus, while our present studies suggest a common mechanism by which HDAC1 protein is depleted, they also suggest that multiple signaling pathways beyond those involved in inflammatory responses can promote HDAC1 depletion. Additionally, our studies indicate that there may be a hierarchy of HDAC1 responses to different signaling pathways, whereby HDAC1 depletion promoted by PN is dominant over any effects on HDAC1 homeostasis caused by the inhibition of IKK2-mediated HDAC1 depletion by PN.

The involvement of ATM in the pathway leading from PN to HDAC1 depletion was rather unexpected. ATM is a PIKK that serves as a DNA-damage transducer. Normally, it is activated by double-strand DNA breaks, though there is evidence that non-DNA damaging agents such as chloroquine and other genotoxic stresses such as ROS can also activate ATM [39, 40]. Since PN is known to increase ROS levels, it is tempting to conclude that this is the mechanism by which PN activates ATM and thereby induces HDAC1 depletion. However, our preliminary data with other agents that modulate ROS levels (e.g., H_2O_2 and ascorbic acid) suggest that increasing ROS alone does not cause HDAC1 depletion (data not shown). Thus, other targets of PN must be involved in this process. Presently, our working hypothesis is that PN chemically modifies a reactive

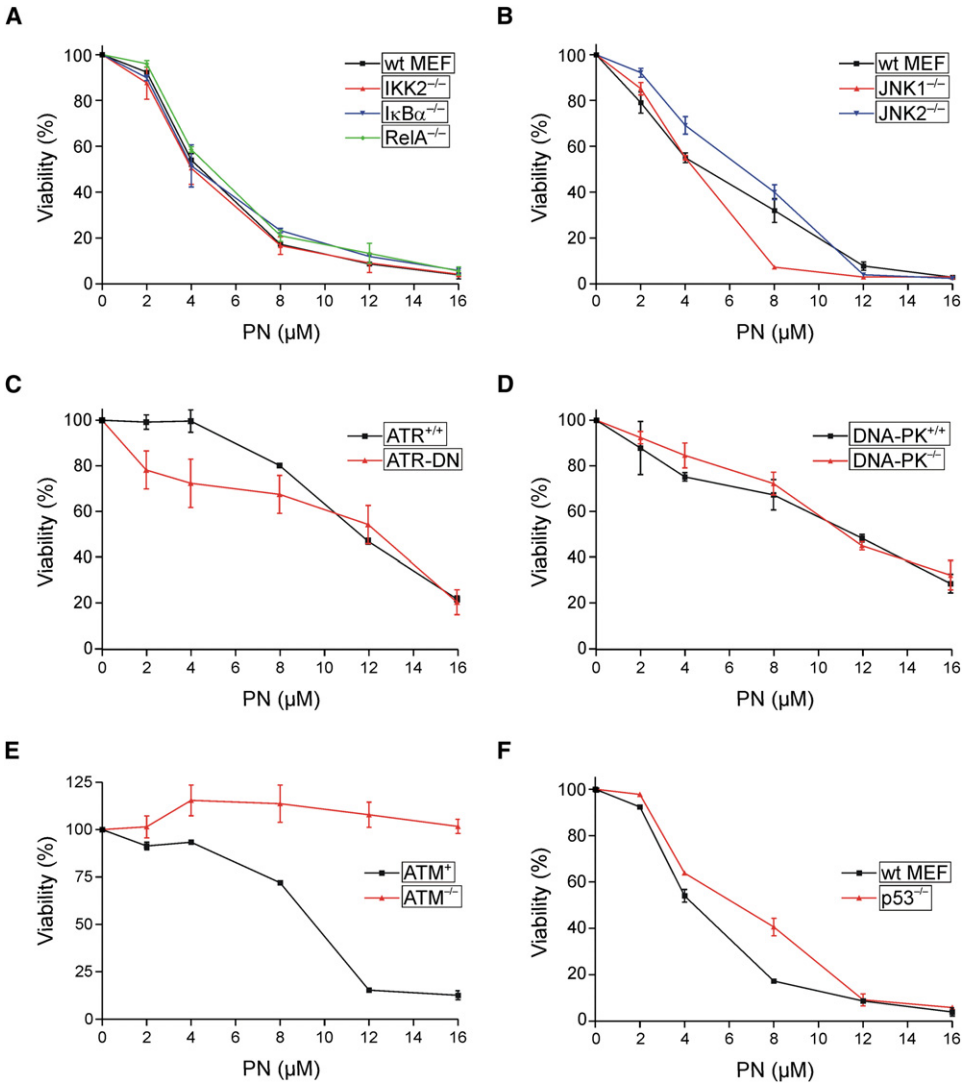

Figure 6. PN Cytotoxicity Requires ATM

(A) Plots of WST-1 viability assays of wild-type (black, squares), IKK2$^{-/-}$ (red, triangles), IκBα$^{-/-}$ (blue, inverted triangles), and RelA$^{-/-}$ (green, diamonds) MEFs treated for 48 hr with the PN concentrations indicated. Error bars indicate standard error of the mean.

(B) Plots of WST-1 viability assays of wild-type (black, squares), JNK1$^{-/-}$ (red, triangles), and JNK2$^{-/-}$ (blue, inverted triangles) MEFs treated for 48 hr with the PN concentrations indicated. Error bars indicate standard error of the mean.

(C) Plots of WST-1 viability assays of uninduced (black, squares) and doxycycline-induced GK41-U2OS cells (red, triangles) expressing a dominant negative mutant (ATR-DN) of the PIKK ATR treated for 48 hr with the PN concentrations indicated. Error bars indicate standard error of the mean.

(D) Plots of WST-1 viability assays of human glioma cells possessing (black, squares) and lacking (red, triangles) the PIKK DNA-PK treated for 48 hr with the PN concentrations indicated. Error bars indicate standard error of the mean.

(E) Plots of WST-1 viability assays of human skin fibroblasts possessing (black, squares) and lacking (red, triangles) the PIKK ATM treated for 48 hr with the PN concentrations indicated. Error bars indicate standard error of the mean.

(F) Plots of WST-1 viability assays of wild-type (black, squares) and p53$^{-/-}$ (red, triangles) MEFs treated for 48 hr with the PN concentrations indicated. Error bars indicate standard error of the mean.

cysteine residue in a thiol-responsive protein, thereby initiating a signaling cascade leading to ATM activation and subsequent downstream effects, including ubiquitination of the lysine-rich C-terminal region of HDAC1, proteasomal degradation of HDAC1, and upregulation of genes normally repressed by elevated levels of HDAC1 (e.g., p21$^{WAF1/CIP1}$). A schematic outlining this pathway is shown in Figure 7. Identification of the proteins involved in this signal transduction pathway, including the putative sensor protein and responsible E3 ubiquitin ligases and deubiquitination enzymes, as well as determining the mechanisms by which PN initiates this process and cells re-establish HDAC1 homeostasis following PN treatment are several major undertakings presently underway in our laboratory.

The cytotoxic effects of PN are apparently mediated through ATM and not through the previously described

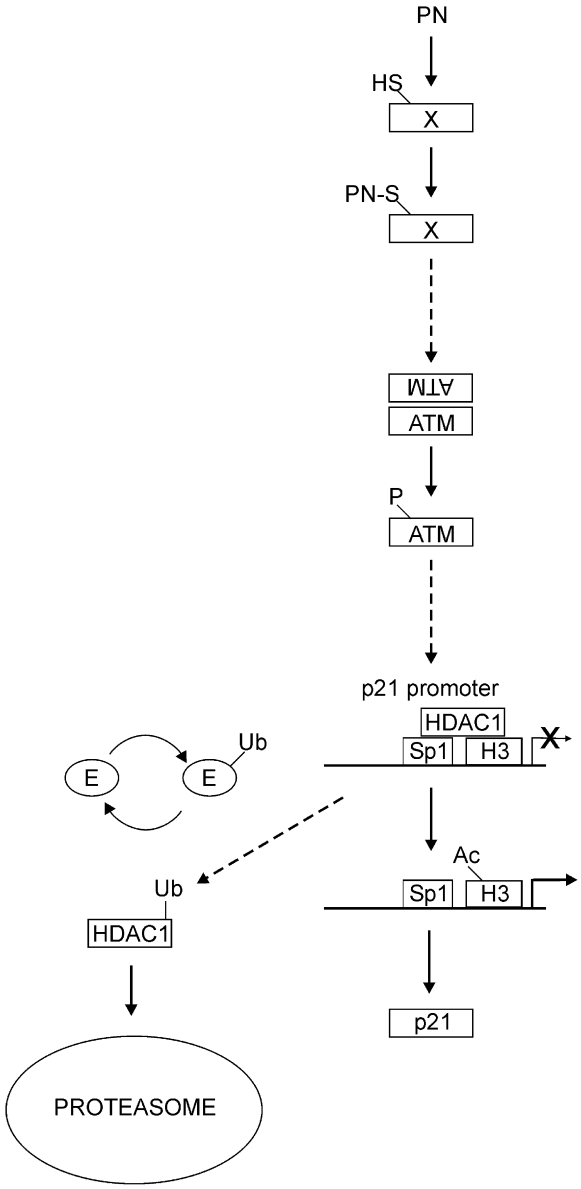

Figure 7. Schematic Representation of the Signal Transduction Pathway that Leads from PN to HDAC1 Depletion and p21 Activation

X, unknown sensor protein; Sp1, transcription factor Sp1; H3, histone H3; E, unknown E3 ubiquitin ligase. HS refers to a free thiol moiety. P, Ub, and Ac refer to phosphorylated, ubiquitinated, and acetylated proteins, respectively. Dotted lines indicate that multiple steps may be involved.

mediators of PN action: IKK2 and JNK [23, 26]. Yet, inhibition of IKK2 is known to promote apoptosis through the inhibition of NF-κB-dependent antiapoptotic gene expression, sustained JNK activation is known to promote apoptosis, and a crosstalk exists between these two signaling pathways [41]. It could very well be that there exists a functional redundancy between individual pathway members (e.g., JNK1 and JNK2) or entire signaling pathways that permits transmission of apoptotic signals prompted by PN. Thus, one can not exclude the possibility that the IKK→IκBα→NF-κB and JNK signaling pathways may be involved PN-mediated cytotoxicity, especially for certain cell types and/or under particular circumstances. Alternatively, additional proapoptotic signaling pathways (e.g., $p38^{MAPK}$) may be involved in PN's mechanism of action, especially in some of the most sensitive cell types (e.g., Jurkat T cell leukemia cell line). Further studies will be required to identify the entire kinome responsible for PN's cytotoxic effects in different cell types.

The absence of HDAC1 in mice is embryonic lethal, resulting from severe proliferation defects and retardation in development [7]. This is thought to result from decreased cyclin-dependent kinase activities and elevated levels of the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. In addition, specific suppression of HDAC1 has been shown to promote apoptosis in melanoma cells through a p53-dependent upregulation of the proapoptotic proteins Bax, Bam, PUMA, and Noxa [42]. While neither cell-cycle arrest nor p53-dependent apoptosis are solely indicated in PN-mediated cytotoxicity, HDAC1 has been suggested to transcriptionally regulate several apoptosis-related genes [30], and suppression of histone deacetylases in general is well known to induce apoptosis [4, 6, 8]. Thus, it is interesting to speculate that the cytotoxic effects of PN are the direct result of HDAC1 depletion, through both p53-dependent and p53-independent mechanisms.

HDACi are a new breed of anticancer agents that induce differentiation, cell-cycle arrest, and apoptosis by upregulation of important cell-cycle regulatory and proapoptotic genes (e.g., p21, p53, and Bax) by both epigenetic and posttranslational mechanisms [4, 8]. Despite their impressive therapeutic properties, these inhibitors nonspecifically inhibit the enzymatic activities of most HDACs and other proteins with similar catalytic domains and induce undesirable toxic effects. More preferable would be molecules that target specific HDACs and especially HDAC1, given its essential role in cell proliferation. Although PN is not a classic HDACi, its ability to deplete HDAC1 protein levels through ubiquitination and proteasomal degradation induces an HDACi-like effect on the p21 gene promoter, namely, increased acetylation of histone H3 and increased transcription of p21. Such is reminiscent of specific HDAC1 depletion through short interfering RNAs [30]. Thus, PN may be considered the first example of a small molecule that specifically inhibits the activity of a single class I HDAC, HDAC1, through modulation of its biological regulatory pathways. Of course, the inhibition of HDAC1 will ultimately have consequences on the activity of other HDACs (e.g., HDAC2), as has been previously observed [30]. However, it is hoped these effects will prove less deleterious to patients than sustained, systemic pan-HDAC inhibition and that molecules like PN will prove useful in the treatment of chronic diseases such as cancer.

It is increasingly being recognized that many, if not all cancers, originate from very small populations of pluripotent, self-renewing cancer stem cells (CSCs) [43, 44]. This follows from observations that large numbers of cancer

cells are normally required to transplant a tumor and that most cancers are heterogeneous and not clonal. In addition, CSCs typically are relatively quiescent, possess long lifespan, have active DNA repair, and are resistant to apoptosis [45]. Thus, CSCs are difficult to eliminate through conventional radiation, hormone, and cytotoxic chemotherapies and are likely causes of cancer recurrence in otherwise successfully treated patients, i.e., those exhibiting reductions in tumor mass or leukemic cell counts. To date, PN is the only small molecule that has been reported to selectively target CSCs [27]. How PN is able to accomplish this is unknown, though is thought to involve both its inhibition of NF-κB activation and its effects on ROS. In light of our data, it is curious to speculate that PN's effects on ATM activation and HDAC1 depletion may also play a role in its effectiveness against CSCs, and suggest additional targets that can be investigated for the development of agents that specifically target CSCs and lead to a durable cure for cancer.

SIGNIFICANCE

PN is a sesquiterpene lactone and the active ingredient in feverfew, an herbal remedy for fever, migraines, arthritis, and superficial inflammation. Its anti-inflammatory properties have been shown to result from its specific inhibition of the signalsome protein IKK2, which is involved in the proinflammatory signal transduction pathway mediated by the transcription factor NF-κB. PN has also been shown to disrupt redox regulation and sustain JNK activation, both leading to cell death. We have found that PN specifically depletes cellular levels of HDAC1 protein through a ubiquitination/proteasomal degradation mechanism that does not require IKK2 or both JNKs but instead requires the PIKK ATM. Similarly, the cytotoxicity of PN was dependent on ATM and not members of either the IKK→IκBα→NF-κB or JNK signaling pathways. Our data suggests that PN has additional cellular targets than IKK2, which play a key role in PN's cytotoxicity. Such information should prove useful in identifying the unique mechanisms by which the small molecule PN specifically targets cancer stem cells, the putative roots of all cancer. In addition, promoting the biological depletion of HDAC1 by PN is a novel approach for the inhibition of specific HDACs, which offers a new paradigm for the epigenetic treatment of cancer and other chronic diseases.

EXPERIMENTAL PROCEDURES

Cell Culture

The ZR-75-1 breast cancer cell line and the transformed human embryonic kidney cell line 293T were obtained from the American Type Culture Collection (Manassas, VA). HCT-116 and HCT116 p53$^{-/-}$ cells were generated by Bert Vogelstein (The Johns Hopkins University School of Medicine) and acquired from Peng Huang (our institution). Wild-type mouse embryo fibroblasts (MEFs) and their complementary RelA$^{-/-}$, IKK2$^{-/-}$, and IκBα$^{-/-}$ cells were acquired from Paul Chiao (our institution). MEFs were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. AT22IJE-T cells (ATM$^{-/-}$ skin fibroblasts) and ATM protein-expressing (ATM^{+}) AT22IJE-TpEBS7-YZ5 cells generated by Yossi Shiloh (Tel Aviv University Sackler Faculty of Medicine) as well as DNA-PK$^{-/-}$ M059J and complementary DNA-PK$^{+/+}$ M059K human glioma cells were acquired from Sai-chin Yeung (our institution). GK41-U2OS human osteosarcoma cells, which inducibly express a dominant-negative, Flag peptide-tagged, kinase-dead ATR protein, were obtained from Paul Nghiem (Harvard Medical School) and induced by using 1 μg/ml doxycycline 45 hr prior to treatment with PN.

Generally, cells were plated at a density of 10^6 cells per 10 cm dish and incubated for 36–48 hr, depending on the cell line used, which resulted in 70%–80% cell confluency in the dishes (~2 × 10^6 cells). The medium was replenished before the drugs were added, and the cells were incubated with the drugs for indicated periods, washed in cold phosphate-buffered saline, and harvested.

Plasmids, Antibodies, and Drugs

Plasmids capable of expressing C-terminal FLAG epitope-tagged wild-type and different mutant HDAC1 proteins were obtained from Ronald DePinho (Dana Farber Cancer Institute). HDAC1, HDAC3, HDAC4, HDAC6, ATM, paxillin, nonimmunogenic control IgG, and poly(ADP-ribose) polymerase antibodies were obtained from Santa Cruz Biotechnology; HDAC2, HDAC8, histone H3, and acetyl histone H3 antibodies were obtained from Upstate Cell Signaling Solutions; DNA-PK antibody was obtained from GeneTex; p21 and ubiquitin antibodies were obtained from Cell Signaling Technology; and FLAG and β-actin antibodies were obtained from Sigma-Aldrich. Chemicals PN, wortmannin, and rapamycin were from Sigma-Aldrich; LY294002 was from Cell Signaling Technology.

Western Blotting

Whole-cell, nuclear, and cytoplasmic extracts were prepared as previously described [19]. Laemmli extracts were prepared by the addition of 2× sample buffer (125 mM Tris-Cl [pH 6.8], 20% glycerol, 2.5% sodium dodecyl sulfate, 10% 2-mercaptoethanol, and 0.005% bromophenol blue) to the cells followed by sonication to shear chromatin. Typically, 50 μg of each extract was resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and blotted with the appropriate antibody. Protein bands were detected by using a SuperSignal chemiluminescence detection kit (Pierce Biotechnology).

Real-Time Reverse-Transcription PCR

RNA extraction and reverse-transcription protocols as well as TaqMan probe-based quantitative RT-PCR analysis of mRNA were described previously [19]. The HDAC1, p21, and GAPDH primer-probe mixes (Taqman Gene Expression Assays, Applied Biosystems) were used according to the manufacturer's instructions.

Immunoprecipitation

Nuclei were isolated from ZR-75-1 cells as previously described [19] and disrupted in RIPA lysis buffer consisting of 50 mM Tris-Cl (pH 7.4), 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin, and aprotinin. The lysates were centrifuged to remove chromatin and the supernatant protein concentration determined using the Bio-Rad Protein Assay. The supernatants were precleared with a nonimmunogenic antibody and protein G-agarose beads (Roche Applied Science) and then incubated with HDAC1 antibody for 12 hr with continuous rotation. The antigen-antibody complexes were bound to protein G-agarose, washed thrice with RIPA buffer, extracted with 2× Laemmli sample buffer, resolved by SDS-PAGE, and western blotted for HDAC1 and ubiquitin.

Transient Transfection

293T cells were grown to 70% confluency in 100 mm tissue culture dishes overnight and transfected with 5 μg of plasmid by using the

Fugene HD reagent (Roche Applied Science) following the manufacturer's instructions. After 36 hr, the cells were treated with PN for 3 hr, and nuclear extracts prepared for analysis by western blotting.

HDAC Activity Assay

HDAC activity was determined with an HDAC colorimetric assay kit (Upstate Cell Signaling Solutions) in 96-well plates. HDAC1 was first immunoprecipitated as described above. Then the antigen-antibody complexes bound to protein G-agarose were resuspended in RIPA buffer and added directly to the assay. Afterwards protein G-agarose and associated proteins were pelleted following centrifugation at 4,000 × g for 1 min, and equal volume supernatants were transferred onto fresh plates, and their absorbance measured. HDAC activity was plotted relative to the activity present in an untreated control.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed with a ChIP assay kit (Upstate Cell Signaling Solutions) as described previously [19]. PCR amplification of the Sp1 binding region (–262 to +31) of the p21 promoter was performed with the primers 21sp-262 (CAGCGCAC CAACGCAGGCG) and 21sp+31 (CAGCTCCGGCTCCACAAGGA). The amplified products were run on a 2% agarose gel and stained with ethidium bromide, and the band densities were determined with an Alpha Innotech gel analysis system.

siRNA Transfection

Transfection of cells with 20 nM ATM SMARTpool siRNA (Dharmacon) was performed by using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated for 48 hr before subsequent drug treatment.

Cytotoxicity Assay

Cytotoxicity of PN and its inhibition of cell proliferation were measured with the WST-1 assay reagent (Roche Applied Science). The cells were seeded in 24-well plates at a concentration of 10,000 cells/well in 500 μl of medium and incubated for 12 hr. Drugs at indicated concentrations were added to the wells, and the cells were incubated for an additional 48 hr. Following the manufacturer's instructions, WST-1 reagent was added to the cells, and after appropriate incubation, the absorbance was measured. The absorbance data were plotted as the percent change of absorbance against the concentration of the drug.

Supplemental Data

Supplemental Data include one figure that is available online at http://www.chembiol.com/cgi/content/full/14/7/813/DC1/.

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