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Parthenolide promotes the ubiquitination of MDM2 and activates p53 cellular functions

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Abstract

MDM2 belongs to a class of ring-finger domain–containing ubiquitin ligases that mediate the proteasomal degradation of numerous proteins, including themselves. Arguably, the most important substrate of MDM2 is p53, which controls cell cycle progression and apoptosis. MDM2 and p53 are parts of a feedback regulatory loop whose perturbations are often present in cancer and are targets for anticancer drug development. We found that the natural product, small-molecule anti-inflammatory agent parthenolide (PN), which is actively being investigated as a potential therapeutic for many human cancers, induces ubiquitination of MDM2 in treated cells, resulting in the activation of p53 and other MDM2-regulated tumor-suppressor proteins. Using cells with functional gene deletions and small interfering RNA knockdown studies, we found that these effects required the DNA damage transducer ataxia telangiectasia mutated. The effects of PN on tumor suppressor activation were comparable with that of nutlin-3a, a recently developed small molecule that was designed to interfere with the interaction between MDM2 and p53 but does not promote MDM2 ubiquitination. Our study illustrates an alternative approach for controlling MDM2 and p53 activities and identifies an additional critically important cancer pathway affected by PN. [Mol Cancer Ther 2009; 8(3):552–62]

Introduction

The p53 tumor-suppressor protein is the ''critical node'' upon which numerous signaling pathways converge to decide cellular fate in response to various stimuli, ranging from housekeeping operations and genotoxic stress to oncogenic stimuli (1, 2). To elicit an effective and timely response to these stimuli, the cellular concentration and transcriptional activity of p53 are tightly maintained through posttranslational modifications. The amount of p53 is primarily regulated by the MDM2 oncoprotein through a negative feedback mechanism, whereby elevated levels of p53 activate MDM2 expression, which in turn sequesters p53, ubiquitinates it, and marks it for proteasomal degradation and/or nuclear exclusion (3, 4). Although MDM2 keeps p53 levels in check, phosphorylation of p53 in its NH_2 terminus by the serine/threonine kinases Chk1, Chk2, and ataxia telangiectasia mutated (ATM) abrogates its interaction with MDM2, thereby increasing p53 stability (5, 6). Acetylation of p53 in its COOH terminus by the acetyltransferases p300/CBP and P/CAF promotes p53 transcriptional activity (7). Reciprocally, MDM2 recruits the lysine deacetylase HDAC1 to the p53-MDM2 complex, thereby deacetylating p53 and promoting its MDM2-mediated ubiquitination (8, 9).

Not surprisingly, MDM2 has been found to be overexpressed in several cancers, where it is responsible for p53 inactivation, increased cell proliferation, and apoptosis resistance (10–13). MDM2 overexpression is associated with metastatic and recurrent cancers (14, 15), is a significant risk factor for distant metastasis (16), and is associated with poor patient prognosis (17). In addition, a growing body of evidence indicates that MDM2 has many p53-independent activities, which can also play important roles in cancer etiology and progression (18). For example, MDM2 overexpression negatively regulates E-cadherin (19), a protein whose loss is associated with cancer progression and metastasis (20). There have been numerous efforts to develop drugs that specifically target the enzymatic activity of MDM2 but with limited success (21, 22). More recently, investigators at Hoffmann-La Roche developed a class of small-molecule MDM2 antagonists, called nutlins, using targeted drug design strategies (23). The most potent of these molecules, nutlin-3a, specifically targets and disrupts the MDM2-p53 interaction, resulting in an increase in cellular p53 protein levels. However, this also results in a potent increase of MDM2 protein levels, induced through the p53-MDM2 feedback pathway (23, 24). The full ramifications of this increase in cellular MDM2 are as yet unknown.

Sesquiterpene lactones are a group of over 4,000 hydrophobic natural compounds found predominantly in the flowers of plants belonging to the *Asteraceae* family (25, 26). Of these, parthenolide (PN), commonly extracted from the European feverfew herb (*Tanacetum parthenium*) and used in traditional remedies for arthritis, headaches, fevers, and local skin irritations (27), is actively being

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investigated for use as an anti-inflammatory and anticancer agent (28, 29). The biological activity of PN is thought to be mediated through its α-methylene-γ-lactone moiety (Fig. 1A), which can covalently bind to sulfhydryl groups within proteins, thereby inactivating them (30). Interfering with nuclear factor-κB (NF-κB) activity through IKK2 inhibition and activation of c-Jun-NH_2-kinase (JNK) signaling have been shown to be major mechanisms for anti-inflammatory and anticancer activities of PN (30–33). Most important, PN has been reported to be selectively effective against leukemia progenitor and stem cell populations, one of the few compounds recognized to affect this critical and often refractory population of cancer cells (34, 35).

We previously showed that PN depletes cellular HDAC1 protein through a mechanism involving HDAC1 ubiquitination and proteasomal degradation, much as we had earlier found in cells treated with different proinflammatory agents (36–38). This loss of HDAC1 consequently resulted in increased acetylation of histone H3 and epigenetic activation of the tumor-suppressor protein $p21^{WAF1/CIP1}$. Interestingly, although this increase in p21 required ATM, it was independent of p53, which is a known transcriptional activator of p21 (39). In the current report, we present our surprising discovery of the action of PN on MDM2. We found that PN treatment of cells induces ubiquitination and proteasomal degradation of MDM2 in an ATM-dependent manner, much like HDAC1, and resulted in the activation of p53 and its downstream apoptotic and cell cycle control proteins. Interestingly, we found that that antagonism of PN of MDM2 activity was distinct from that of the MDM2-targeted agent nutlin-3a, which had been under development as an anticancer therapeutic. Our current study suggests that the unique mechanism of action of PN should be explored for clinical cancer therapy.

Materials and Methods

Cell Culture

The ZR-75-1 breast cancer cell line was obtained from the American Type Culture Collection. HCT-116 and HCT-116 $p53^{-/-}$ cells were generated by Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD) and acquired from Peng Huang (our institution). Wild-type, $p53^{-/-}$, and $p53^{-/-}$ $MDM2^{-/-}$ mouse embryo fibroblasts (MEF) were obtained from Guillermina Lozano (our institution); $JNK1^{-/-}$ and $JNK2^{-/-}$ MEFs were obtained from Michael Karin (University of California at San Diego, San Diego, CA); and $RelA^{-/-}$, $IKK2^{-/-}$, and $I\kappa B\alpha^{-/-}$ MEFs were acquired from Paul Chiao (our institution). MEFs were propagated in DMEM containing 10% fetal bovine serum. AT22IJE-T cells ($ATM^{-/-}$ skin fibroblasts) and ATM protein-expressing (ATM^{+}) AT22IJE-TpEBS7-YZ5 cells were generated by Yossi Shiloh (Tel-Aviv University Sackler Faculty of Medicine, Tel Aviv, Israel) and acquired from Sai-chin Yeung (our institution).

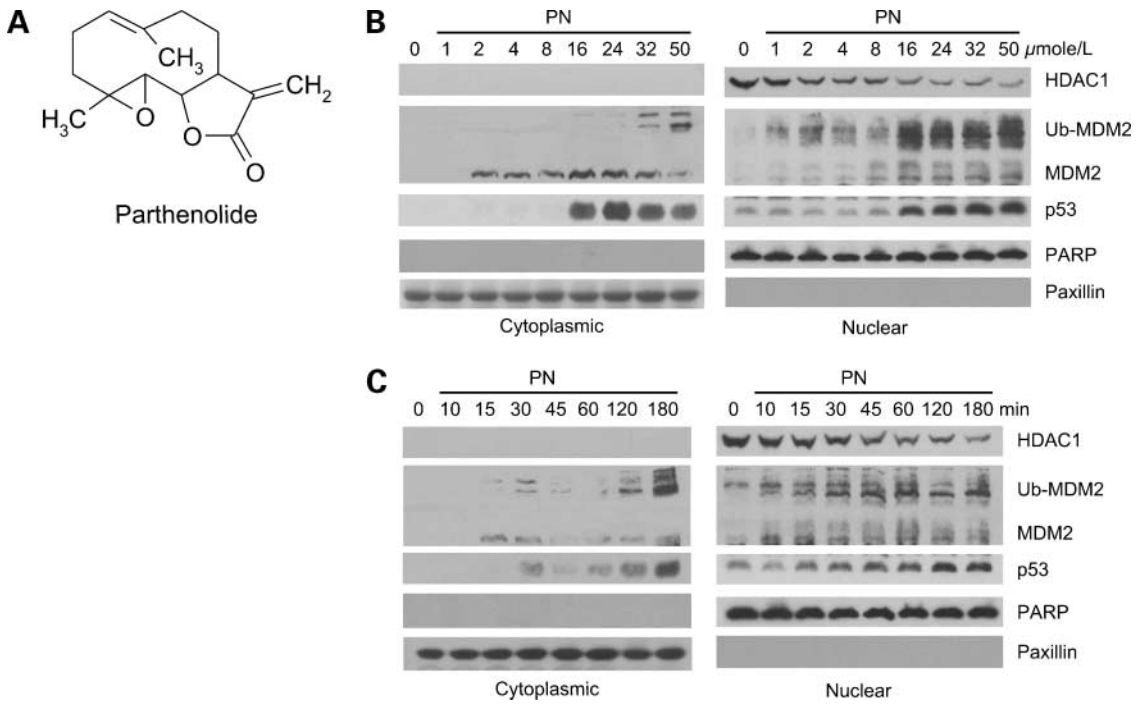

Figure 1. The sesquiterpene lactone PN up-regulates MDM2 and p53 proteins. **A,** chemical structure of PN. **B,** Western blots of cytoplasmic and nuclear extract proteins from ZR-75-1 cells treated with PN at increasing concentrations for 3 h and probed with the indicated antibodies (*right*). Paxillin and poly(ADP-ribose) polymerase (*PARP*) immunostaining served as protein loading controls and as controls for cytoplasmic and nuclear extract integrity. **C,** Western blotting done as described in **A** but with 15 μmol/L PN and increasing treatment durations.

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Antibodies and Drugs

The HDAC1, MDM2, p53, poly(ADP-ribose) polymerase, paxillin, p300, ATM, and E-cadherin antibodies were obtained from Santa Cruz Biotechnology; the ubiquitin, phospho-p53, acetyl-p53, p21, and PUMA antibodies were obtained from Cell Signaling Technology; and the β-actin antibody was obtained from Sigma-Aldrich. Chemicals PN, Z-VAD-FMK, MG-132, wortmannin, and nutlin-3a were from Sigma-Aldrich. The PN used in our studies had a purity of 90%. However, we have also tested 95% and 98% pure PN from other sources and found no differences in the molecular effects of these preparations. PN was dissolved in DMSO and aliquots stored at −80°C before being appropriately diluted in culture medium just before use.

Protein Extraction and Western Blotting

Nuclear, cytoplasmic, and whole-cell extracts; SDS-PAGE; and Western blotting were carried out as previously described (37). Typically, 50 μg of extracts were resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, blotted with the appropriate antibody, and detected by chemiluminescence.

Real-time Reverse Transcription PCR

RNA extraction and reverse transcription protocols as well as TaqMan probe-based quantitative reverse transcription-PCR (qRT-PCR) analysis of mRNA were described previously (37). The p53, MDM2, and glyceraldehyde-3-phosphate dehydrogenase 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 (37) and disrupted in radioimmunoprecipitation assay lysis buffer consisting of 50 mmol/L Tris-Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL each of leupeptin, pepstatin, and aprotinin. The lysates were centrifuged to remove chromatin and the supernatant protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). The supernatants were precleared with a nonimmunogenic antibody and Protein G–Agarose beads (Roche Applied Science) and then incubated with MDM2 or p53 antibodies for 12 h with continuous rotation. The antigen-antibody complexes were bound to Protein G–Agarose, washed thrice with radioimmunoprecipitation assay buffer, extracted with 2× Laemmli sample buffer, resolved by SDS-PAGE, and Western blotted with the described antibodies.

Luciferase Reporter Assay

The pBV-Luc–based reporter plasmids containing four copies of a p53 binding segment from the PUMA gene promoter (BS2wt) or four copies of a mutated p53-binding segment (BS2mut) have been described previously (40) and were obtained from Bert Vogelstein. Plasmid transfections of cells were carried out using the Fugene 6 reagent (Roche Applied Science) and luciferase reporter assays were done using the Dual Luciferase Reporter Assay kit (Promega Corporation) following the manufacturers' protocols.

Small Interfering RNA Transfection

Transfection of cells with 20 nmol/L ATM or HDAC1 SMARTpool small interfering RNA (siRNA; Dharmacon) was done using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated for 48 h and then treated with or without 15 μmol/L PN for 3 h before harvesting.

Cytotoxicity and Apoptosis Assays

Inhibition of cell proliferation by PN was measured using the WST-1 assay reagent (Roche Applied Science) and the experiment was carried out as described earlier (36). The absorbance data were plotted as the percent change of absorbance against the concentration of drug. The apoptosis assay was carried out using the Promega DeadEnd Fluorometric TUNEL Assay kit (Promega Corporation). Briefly, the cells were plated to 60% confluency in 100-mm dishes, allowed to attach overnight, and treated with PN for the required time periods. The cells were trypsinized, fixed in formaldehyde, and subjected to terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) following the manufacturer's protocol and analyzed using a BD FACSCalibur flow cytometer. Similarly, PN-treated, formaldehyde-fixed cells were subjected to propidium iodide staining and the percentage of cells accumulating in the sub-G_1 phase of the cell cycle was determined through fluorescence-activated cell sorting.

Results and Discussion

PN Treatment Promotes Accumulation of p53 and Modified MDM2

We previously showed that PN depleted cellular HDAC1 levels through an ATM-dependent mechanism (36). In addition, ATM is well known to activate p53 by phosphorylating several residues on p53, leading to its dissociation from MDM2 (6). To determine the effects of PN on p53 and MDM2, ZR-75-1 human breast cancer cells, which express a functional, wild-type p53 protein (41), were treated with different concentrations of PN for 3 hours (Fig. 1B) or with 15 μmol/L of PN for different treatment durations (Fig. 1C). Nuclear and cytoplasmic extracts were prepared and changes in protein amounts or electrophoretic mobility were determined by Western blotting. We found that PN induced a rapid increase of p53 protein in a concentration- and time-dependent manner. An increase in cytoplasmic p53 was detected after only 30 minutes of treatment with 15 μmol/L PN, with robust p53 levels being observed after 3 hours together with a corresponding increase in nuclear p53. In the case of MDM2, extended (3 hours) treatment with low (1–2 μmol/L) PN concentrations or brief (10–15 minutes) treatments with intermediate (15 μmol/L) PN concentrations yielded an accumulation of cytoplasmic MDM2 and a substantial increase in a slower mobility species that cross-reacted with smp14, a monoclonal antibody directed against amino acids 154 to 167 of MDM2. This species was not significantly increased

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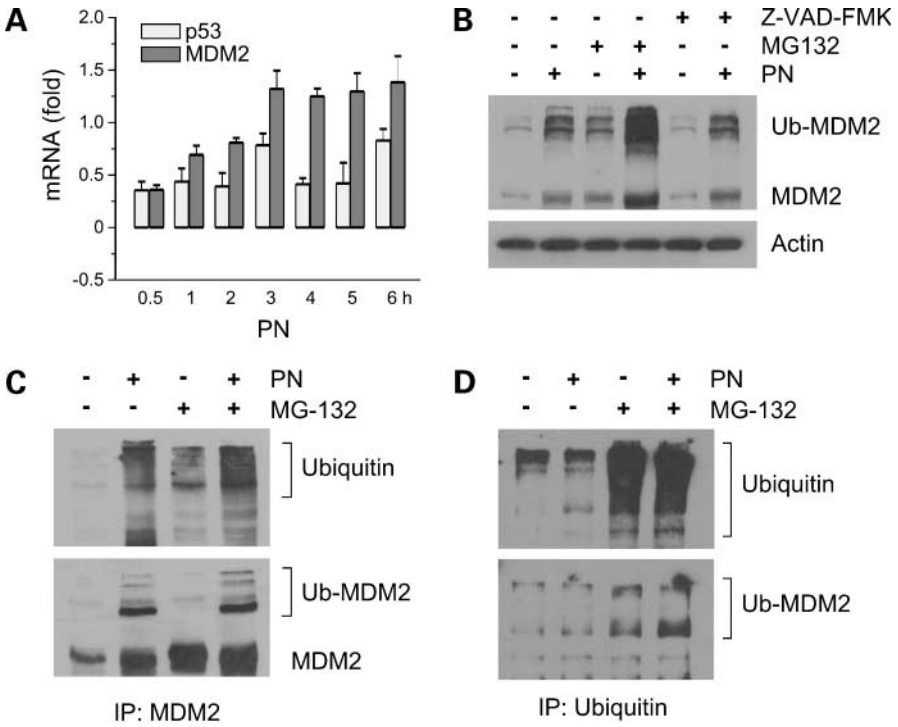

Figure 2. PN causes the accumulation of MDM2 mRNA and ubiquitinated MDM2 protein. **A,** p53 and MDM2 mRNA levels in ZR-75-1 cells treated with 15 μmol/L PN as determined using a real-time qRT-PCR assay after normalization against glyceraldehyde-3-phosphate dehydrogenase mRNA levels. **B,** Western blots of whole-cell extract proteins from ZR-75-1 cells treated for 3 h with 20 μmol/L MG-132, 10 μmol/L Z-VAD-FMK, and 15 μmol/L PN as indicated. **C,** Western blots of MDM2 protein immunoprecipitated from ZR-75-1 cells treated with 15 μmol/L PN and/or 10 μmol/L MG-132 for 3 h, as indicated, and probed with ubiquitin antibodies (*top*) or stripped and reprobed with MDM2 antibodies (*bottom*). **D,** Western blotting as described in **C**, except that the immunoprecipitation was with anti-ubiquitin antibodies.

in abundance when cells were treated with the cytotoxic anticancer agents, camptothecin, cisplatin, and doxorubicin, which are known to increase nuclear p53 protein levels (Supplementary Fig. S1).[1] Notably, these concentration- and time-dependent changes in MDM2 coincided with the PN-dependent depletion of HDAC1 (36), suggesting related causes for these effects. Finally, these changes in MDM2 were observed in several cell lines from different cancer origins (Supplementary Fig. S2),[1] suggesting that PN-mediated MDM2 modification is a general phenomenon using common signal transduction pathways.

Transcriptional and Posttranslational Effects of PN on p53 and MDM2

To address whether PN-mediated up-regulation of p53 and MDM2 proteins was occurring through transcriptional or posttranslational processes, p53 and MDM2 mRNA levels in ZR-75-1 cells were determined using real-time quantitative reverse transcription-PCR. We found that p53 mRNA levels changed in a cyclic fashion, with peak levels being observed at 3 and 6 hours after PN treatment (Fig. 2A). This change was not consistent with the substantial increase in p53 protein observed after PN treatment (Fig. 1C), suggesting that posttranscriptional processes were primarily responsible. In contrast, MDM2 mRNA levels increased linearly to a maximum of 3 hours after PN treatment and remained at this high level for an additional 3 hours (Fig. 2A). Such changes could explain, at least in part, the overall increase in MDM2 protein observed after PN treatment. It is not known whether these changes in mRNA levels reflected increased transcription or mRNA stability. However, the real-time quantitative reverse transcription-PCR data for MDM2 are consistent with increased transcription, given that p53 is a known transcriptional activator of the MDM2 promoter (42).

The slower mobility species observed after PN treatment is consistent with a posttranslational modification of MDM2. The rather large decrease in apparent electrophoretic mobility (>10 kDa) and banding pattern suggest the addition of multiple and/or high-molecular weight modifications (e.g., SUMOylation, NEDDylation, and ubiquitination). MDM2 is well known to be ubiquitinated through its own E3 ubiquitin ligase activity, leading to its inactivation and degradation (3). To determine whether the higher mobility bands induced by PN treatment represented ubiquitinated MDM2, ZR-75-1 cells were treated with the proteasomal inhibitor MG-132 or with the caspase inhibitor Z-VAD-FMK. Treatment with MG-132 alone or PN alone increased the apparent amounts of both unmodified and modified MDM2, which suggests that PN may either inhibit the proteasomal degradation of MDM2 or increase the ubiquination of MDM2 (Fig. 2B). Z-VAD-FMK treatment had no apparent effect on either MDM2 species, suggesting that caspases are not involved in PN-mediated MDM2 changes. To confirm whether the modification of MDM2 is ubiquitination, whole-cell extracts from cells treated with PN and/or MG-132 were immunoprecipitated with either anti-MDM2 or anti-ubiquitin antibodies, resolved by SDS-PAGE, and subsequently Western blotted with either anti-ubiquitin or anti-MDM2 antibodies (Fig. 2C and D, *top* and *bottom*, respectively). We found that PN and

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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MG-132 treatment led to an additive increase in ubiquitinated species as well as in unmodified MDM2, which was not observed when a nonimmunogenic control antibody was used (Supplementary Fig. S3).[1] These data confirmed ubiquitination of MDM2. Probing these immunoprecipitates with SUMO-1, SUMO-2/3, or SUMO-4 antibodies or a NEDD antibody provided no evidence that the modified proteins were SUMOylated or NEDDylated MDM2 (data not shown).

Parthenolide Disrupts the Interaction of MDM2 and HDAC1 with p53 and Activates p53

Because MDM2 recruits HDAC1 to p53, which in turn deacetylates p53 and promotes its inactivation by MDM2 (8, 9), we hypothesized that PN treatment may conversely activate p53 through degradation of p53-associated MDM2 and HDAC1. To test this hypothesis, nuclear extracts from PN-treated ZR-75-1 cells were immunoprecipitated with anti-p53 antibodies and the associated proteins were probed by Western blotting. We observed that PN treatment increased the levels of immunoprecipitated p53 (Fig. 3A), which was consistent with the increase of total p53 observed in the input extracts. This increase was accompanied by dissociation of p53-bound HDAC1 as well as ubiquitination of p53-bound MDM2 within 30 minutes of PN treatment. However, dissociation of MDM2 from p53 was slower than HDAC1 dissociation and occurred over 3 hours. Finally, there was a temporal increase in the association of the histone acetyltransferase protein, p300, with p53. Dissociation of HDAC1 followed by association of p300 would strongly suggest that p53 should be acetylated after PN treatment. Similarly, MDM2 dissociation from p53 should result in p53 phosphorylation at serine-15. Western blotting of lysates from PN-treated cells showed increases in p53 acetylation at lysine-382 and phosphorylation at serine-15 (Fig. 3B), which are posttranslational modifications corresponding to transcriptionally active p53 (43).

Further evidence of p53 activation by PN could be found in the increased expression of PUMA and $p21^{WAF1/CIP1}$ proteins (Fig. 3C), both of whose promoters are transcriptionally up-regulated by p53 (39, 40, 44). Note that the increase in PUMA was only observed in PN-treated cell lines containing wild-type p53 (ZR-75-1 and HCT-116) and not in one lacking p53 (HCT-116 $p53^{-/-}$). Such was not the case for p21, which can be transcriptionally activated by a decrease in HDAC1 levels independent of p53 status of cells (36). Finally, proof that PN increased the transcriptional activity of p53 was found through transient transfection experiments with a p53-responsive reporter plasmid containing four p53 binding sites from the PUMA gene promoter (BS2wt) and a corresponding p53-unresponsive plasmid containing mutated p53 binding sites (BS2mut). We observed a 4- to 8-fold increase in luciferase activity only from cells transfected with BS2wt after PN treatment (Fig. 3D). Taken together, our data show that PN treatment releases MDM2 and HDAC1 from p53, resulting in its increased transcriptional activity.

One of the important transcriptional targets of activated p53 is its negative regulator, MDM2 (42). MDM2 up-regulation by p53 provides an important negative feedback control over cellular p53 levels and activity. We hypothesized that such a feedback control was responsible for our observed increase in unmodified MDM2 levels rather than its depletion after proteasomal degradation. We tested this hypothesis using MEF cell lines possessing wild-type or homozygous deletions of the murine p53 genes. We found that PN treatment resulted in an increase of unmodified MDM2 in cells containing wild-type p53 but not in $p53^{-/-}$ cells (Fig. 3E). In $p53^{-/-}$ cells, accumulation of ubiquitinated MDM2 peaked at 60-minute exposure and decreased rapidly thereafter. Thus, wild-type p53 was responsible, at least in part, for the maintenance of observed elevated levels of ubiquitinated MDM2. The disappearance of the ubiquitinated MDM2 bands in the $p53^{-/-}$ cells suggests that ubiquitination of MDM2 by PN treatment leads to its proteasomal degradation. More important, these data also showed that p53 activity was not required for PN-mediated MDM2 degradation. This is an important aspect of the action of PN because MDM2 itself is a worthwhile anticancer target, even in the absence of wild-type p53, and suppression of MDM2 has been found to promote apoptosis in cell lines containing mutant p53 (24).

Mechanism of MDM2 Ubiquitination and p53 Activation by PN

PN has been previously described to specifically inhibit the signalsome protein IKK2, thereby preventing NF-κB activation and its program of antiapoptotic gene expression (30). PN has also been found to activate the JNK kinases, thereby leading to apoptotic cell death (28, 33). However, studies using a panel of MEFs harboring homozygous deletions of different JNK or IKK/NF-κB signal transduction pathway members strongly suggested that neither the IKK/NF-κB or JNK pathways are required for the effect of PN on MDM2 (Supplementary Fig. S4).[1]

We previously found that the DNA damage transducer ATM is required for depletion of HDAC1 after PN treatment, as well as the cytotoxic effects of PN (36). ATM is a well-known activator of p53, a process integral to its function in DNA damage response (45). However, we found through a γ-H2AX immunocytochemical staining experiment that the activation of ATM by PN did not involve substantial DNA damage (Supplementary Fig. S5).[1] ATM achieves p53 activation through multiple phosphorylation events, including phosphorylation of p53 as well as its inhibitor protein MDM2 (45–47). Thus, it would be reasonable to assume that ATM is involved in the ubiquitination and proteasomal degradation of MDM2 caused by PN treatment. Using chemical kinase inhibitors, we found that high (20 μmol/L) but not low (1 μmol/L) concentrations of wortmannin inhibited both MDM2 ubiquitination and p53 accumulation (Fig. 4A). These data are consistent with the involvement of a phosphoinositol-3-kinase–like kinase (PIKK), of which ATM is an example. More concrete proof was obtained using paired human skin fibroblast cell lines either lacking or possessing wild-type ATM protein. With these, ATM was absolutely required for both the accumulation of unmodified

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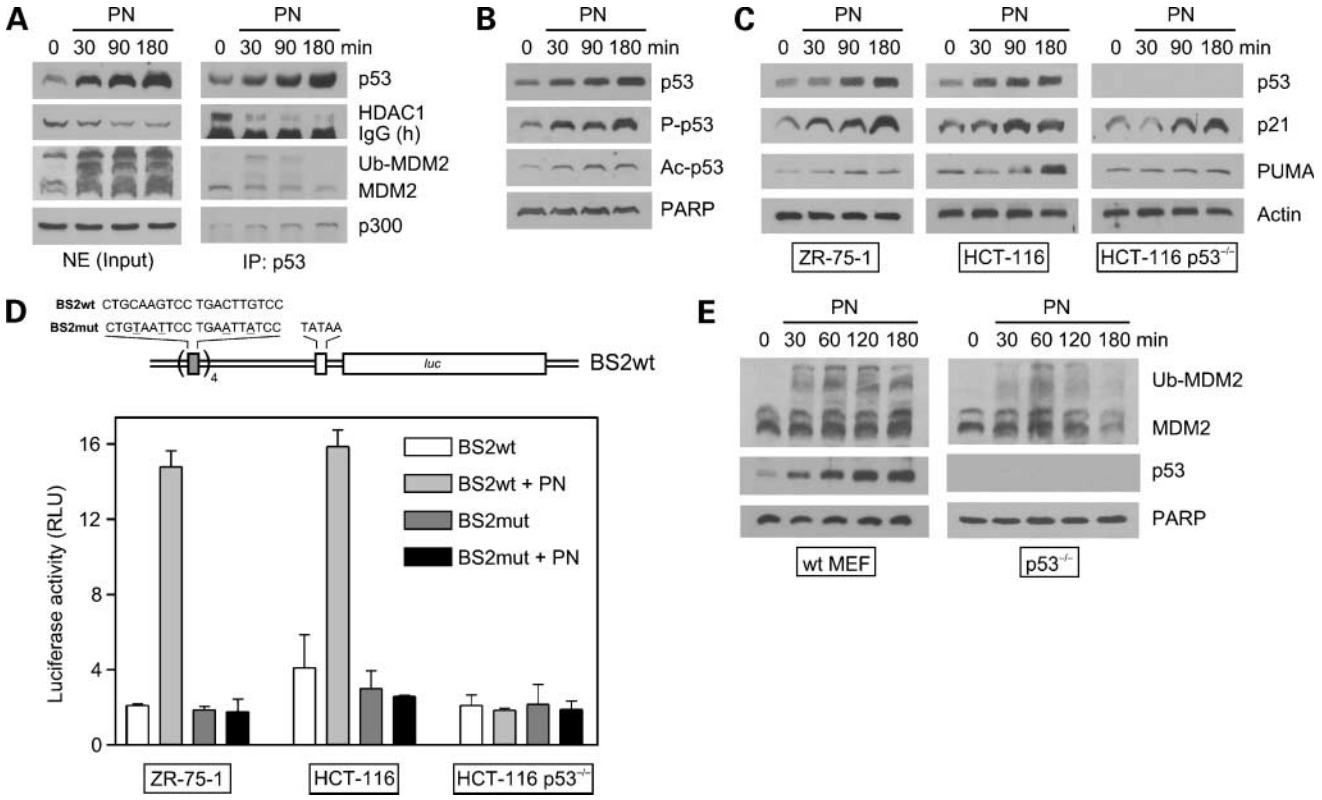

Figure 3. PN promotes MDM2-p53 complex dissociation, p53 activation, and p53-independent MDM2 degradation. **A,** Western blots of input nuclear extracts (*NE*) and corresponding p53 immunoprecipitated proteins from ZR-75-1 cells treated with 15 μmol/L PN for the times indicated and probed with the antibodies indicated at right. **B,** Western blot of nuclear extract proteins from ZR-75-1 breast cancer cells treated with 15 μmol/L PN. **C,** Western blots of whole-cell extracts from wild-type p53-containing cells (ZR-75-1, HCT-116) and p53-deficient cells (HCT-116 $p53^{-/-}$) treated with 15 μmol/L PN. **D,** *top,* schematic representation of pBS2 reporter plasmids containing four copies of either wild-type (*wt*) or mutant (*mut*) p53 binding sites, a TATA-box minimal promoter, and the luciferase gene (*luc*). *Bottom,* graph of p53-responsive (*BS2wt*) and control (*BS2mut*) firefly luciferase activity in ZR-75-1, HCT-116, and HCT-116 $p53^{-/-}$ cells transfected with the aforementioned plasmids for 36 h and then treated for 5 h with 15 μmol/L PN. Values are presented in relative luminometer units (*RLU*) following normalization of firefly luciferase activity to that of *Renilla* luciferase activity expressed from a cotransfected plasmid (pRL-TK) that served as an internal control. **E,** Western blots of nuclear extract proteins from wild-type and $p53^{-/-}$ MEFs treated with 15 μmol/L PN.

and ubiquitinated MDM2 after PN treatment, as well as the accumulation of nuclear p53 (Fig. 4B). Similar results were not observed with cell lines deficient in functional ATR and DNA-PK, suggesting these PIKKs are not involved in mediating the effects of PN on p53 and MDM2 (Supplementary Fig. S6).[1] Our findings with ATM could also be recapitulated in ZR-75-1 cells using siRNA targeted against ATM mRNA (Fig. 4C). Note that although a substantial decrease in cellular ATM levels could be achieved through siRNA treatment, the remaining ATM was sufficient to yield a small increase in nuclear p53 levels, as well as in ubiquitinated MDM2. These data suggest that PN is capable of eliciting and maintaining activity of ATM, even when relatively little ATM is present.

Our Western blotting data in Fig. 1C indicated a temporal correlation between HDAC1 depletion and MDM2 ubiquitination upon treatment with PN. Our immunoprecipitation data from Fig. 3A also indicated kinetically comparable dissociation rates of HDAC1 and MDM2 from p53 after PN treatment. These findings suggested to us that HDAC1 may be important for the regulation of p53-MDM2 homeostasis. To test this hypothesis, we treated ZR-75-1 cells with HDAC1-specific siRNA or a RISC-free control siRNA and incubated the cells for 48 hours. We then subjected these cells to a time course of PN treatments as before. Western blotting of cell extracts revealed that HDAC1 siRNA depleted ~70% of the cellular HDAC1 protein present in treated cells but had only small effects on basal p53, MDM2, p21, and PUMA protein levels (Fig. 4D). Subsequent addition of PN resulted in increased HDAC1 depletion and increases in p53, MDM2, p21, and PUMA protein levels, as previously observed. Similar increases were also observed with p21 and PUMA mRNAs (Fig. 4E). Most notable, in all cases, the effects of HDAC1 siRNA and PN treatments seemed only additive. Taken together, our data indicate that although HDAC1 may be a negative regulator of p53, its absence does not appreciably alter p53-MDM2 homeostasis. However, it remains quite interesting that PN treatment causes p53 activation through cooperative effects on two different negative regulators of p53, MDM2 and HDAC1.

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PN Decreases the Proliferation of Cancer Cells in a Partially p53-Dependent Manner

Our findings suggest that p53 plays an important role in the molecular actions of PN, although p53-independent processes have also been shown by us and by others (29–33, 36). To determine the cellular consequences of p53-dependent and p53-independent processes on the function of PN, WST-1 viability assays were done to determine the effects of PN on the proliferation of matched cell lines possessing (HCT-116) or lacking (HCT-116 $p53^{-/-}$) wild-type p53. After 48 hours of PN treatment, we found that the viability of cells containing wild-type p53 was less than that of p53-deficient cells, especially at intermediate PN concentrations (Fig. 5A). These data were found to be statistically significant through a two-tailed, equal variance *t* test, with $P < 0.05$ for 4 μmol/L PN and $P < 0.01$ for 8, 12, and 16 μmol/L PN. Such observations are similar to those observed with the cytotoxic anticancer agent doxorubicin, which exhibits increased sensitivity in cells possessing wild-type p53 (Supplementary Fig. S7).[1] Our observations concurred with our previous findings that wild-type MEFs were more sensitive to PN than their $p53^{-/-}$ counterparts (36) and suggested that p53 plays an important role in the cytotoxicity of PN. Because p53 is a known mediator of

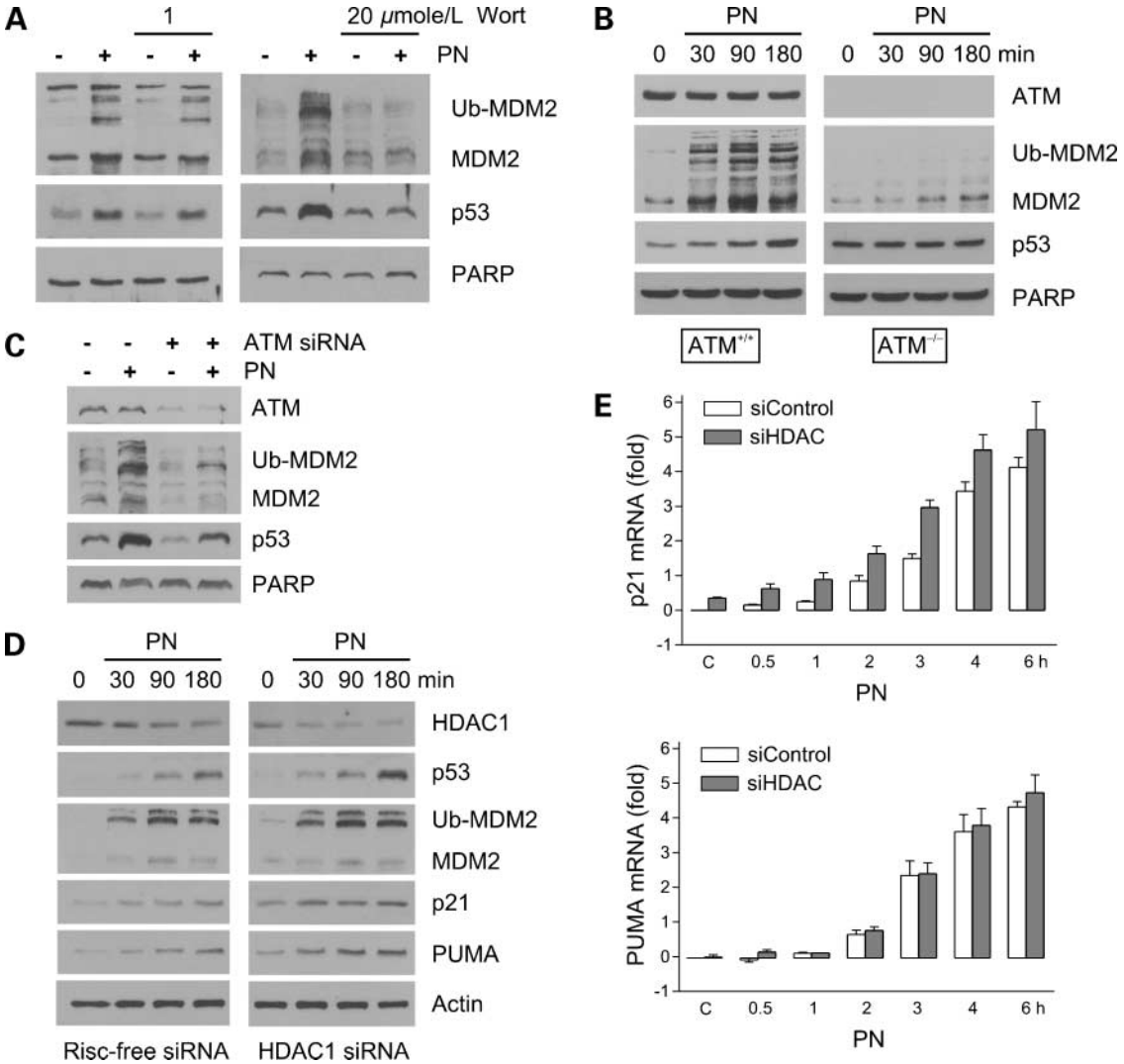

Figure 4. Accumulation of ubiquitinated MDM2 following PN treatment requires the PIKK ATM. **A,** Western blots of nuclear extract proteins from ZR-75-1 cells treated with 15 μmol/L PN and 1 μmol/L or 20 μmol/L wortmannin (*Wort*) for 3 h as indicated. **B,** Western blots of nuclear extract proteins from cells possessing (AT22IJE-TpEBS7-YZ5, *left*) or lacking (AT22IJE, *right*) ATM treated with 15 μmol/L PN. **C,** Western blots of nuclear extract proteins from ZR-75-1 cells treated with ATM siRNA for 72 h and 15 μmol/L PN for the final 3 h, as indicated. **D,** Western blots of whole-cell extract proteins from ZR-75-1 cells treated with control RISC-free siRNA (*left*) or HDAC siRNA (*right*) for 72 h and 15 μmol/L PN for the final durations indicated. **E,** p21 (*top*) and PUMA (*bottom*) mRNA levels in ZR-75-1 cells treated with control RISC-free siRNA (*white columns*) or HDAC siRNA (*gray columns*) for 72 h and 15 μmol/L PN for the final durations indicated, as determined using a real-time qRT-PCR assay after normalization against glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

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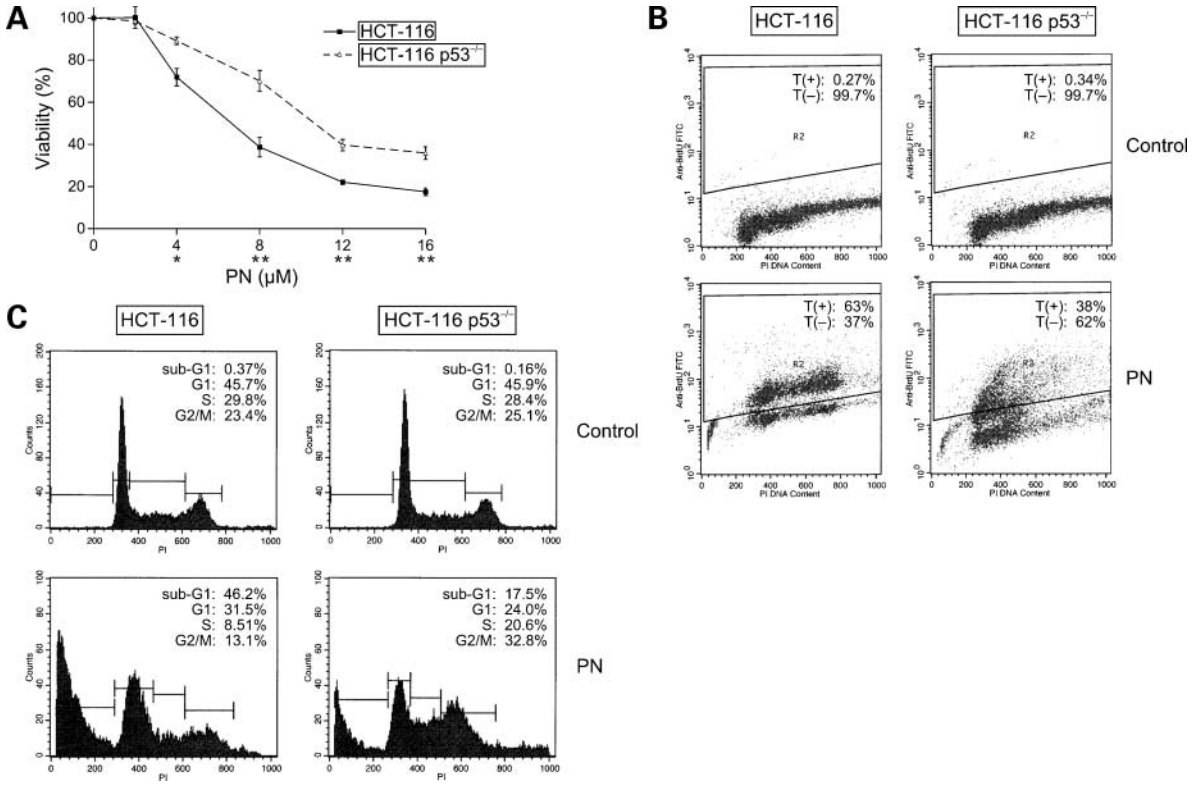

Figure 5. Cytotoxicity of PN is partly dependent on p53. **A,** plots of WST-1 viability assays of HCT-116 (*solid line*, ■) and HCT-116 $p53^{-/-}$ (*dotted line*, △) cells treated for 48 h with the PN concentrations indicated. Statistical significance of differences in viability between the two cell types following PN treatment is indicated as * ($P < 0.05$) and ** ($P < 0.01$) for individual PN concentrations. **B,** TUNEL apoptosis assays of HCT-116 and HCT-116 $p53^{-/-}$ cells treated for 24 h with 15 μmol/L PN. The percentage of total cells present in each field is indicated at the top right of the panel. **C,** FACS cell cycle analysis of similarly treated cells as in **B**. The percentage of total cells present in each cell cycle phase is indicated at the top right of the panel.

programmed cell death or apoptosis (1), we sought to determine whether the decreased viability of wild-type p53 cells was the result of increased apoptosis. To determine this, we carried out a TUNEL assay on the HCT-116 cells and the HCT-116 $p53^{-/-}$ cells after treating them with PN. Fluorescence-activated cell sorting analysis of these cells showed that 63% of the HCT-116 cells and 38% of the HCT-116 $p53^{-/-}$ cells labeled positively for TUNEL after PN treatment (Fig. 5B). The TUNEL-positive $p53^{+/+}$ cells had a fairly uniform intensity profile, which suggested a single dominant mechanism of apoptosis induction, potentially one mediated by p53. On the other hand, the $p53^{-/-}$ cells showed a slightly varied TUNEL intensity, different from that observed with $p53^{+/+}$ cells, which is suggestive of different apoptotic mechanisms. To validate these findings, a propidium iodide dye–based cell cycle profile of the PN-treated cells was generated by fluorescence-activated cell sorting. Cells in the late phases of apoptosis typically accumulate in the sub-G_1 phase of the cell cycle. The data from this experiment showed that 46.2% of the HCT-116 cells and 17.5% of the HCT-116 $p53^{-/-}$ cells accumulated in the sub-G_1 phase upon PN treatment (Fig. 5C). Although the numbers of apoptotic cells in the sub-G_1 assay are lower compared with those from the TUNEL assay, the differences between the $p53^{+/+}$ and $p53^{-/-}$ cells are proportionately similar in both assays. The differences in the apoptotic cell numbers between the two assays may reflect the efficacy of each assay system to detect apoptotic cells. In conclusion, the higher number of PN-treated $p53^{+/+}$ cells in sub-G_1 compared with comparably treated $p53^{-/-}$ cells confirms our TUNEL results and clearly indicate that p53-dependent apoptosis is an important cause underlying the cytotoxicity of PN.

Comparison of the Molecular and Cellular Effects of PN with the MDM2-Targeted Small Molecule Nutlin-3a

About half of all human cancers express a wild-type p53 protein, which is often inactivated by MDM2 sequestration. Hence, the MDM2-p53 nexus is an important target for therapeutic intervention. Several pharmacologic approaches have been explored to disrupt the MDM2-p53 interaction. The most promising of these has been the small molecule nutlin-3a, which binds to the hydrophobic pocket in MDM2 normally occupied by the transactivation domain of p53 (23, 24). This prevents p53 from binding MDM2 and results in p53 accumulation and increased expression of p53-responsive genes, including *p21* and *MDM2*. Thus, nutlin-3a is being explored as a potential chemoprotective and anticancer therapeutic agent.

This mechanism by which nutlin-3a inactivates MDM2 differs from that used by PN, which apparently affects

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endogenous biological regulation of MDM2 ubiquitination and proteasomal degradation. To better understand the differences between these two approaches for MDM2 inactivation and p53 activation, we examined the molecular effects of PN and nutlin-3a treatment on wild-type and MDM2-deficient MEFs. Note that MEFs lacking MDM2 also lacked p53, as this is necessary for their proliferation in culture (48). Wild-type MEFs treated with PN exhibited a rapid increase in ubiquitinated MDM2 (Fig. 6A). In contrast, wild-type MEFs treated with nutlin-3a exhibited a rapid increase of unmodified MDM2 (Fig. 6B). Both treatments yielded similar increases in p53. Additionally, by definition, these changes were dependent on MDM2 and p53. Thus, although both nutlin-3a and PN can similarly activate p53, subsequent transcriptional up-regulation of MDM2 expression by wild-type p53 has two very different outcomes after these treatments—either increased levels of unmodified MDM2 or increased levels of ubiquitinated MDM2.

In cells, p53 is not the only protein affected by MDM2. For example, the cell motility protein E-cadherin was recently shown to be a target of MDM2-mediated proteasomal degradation, whose tumor suppressor function is inhibited in MDM2-overexpressing cells (19). We treated $p53^{-/-}$ $MDM2^{-/-}$ MEFs and their complementary $p53^{+/+}$ $MDM2^{+/+}$ wild-type MEFs with PN or nutlin-3a and analyzed the cell lysates for E-cadherin protein by Western blotting. Both PN and nutlin-3a treatment increased cellular levels of E-cadherin protein in the wild-type cells; this effect was dependent on MDM2 (Fig. 6A and B). Note that the effects of PN and nutlin-3a were not completely identical; for example, PN caused a slight decrease in E-cadherin levels in $p53^{-/-}$ $MDM2^{-/-}$ MEFs, whereas nutlin-3a did not. Taken together, these data suggest that both PN and nutlin-3a can affect p53-independent processes similarly, albeit in certain contexts.

At a molecular level, PN and nutlin-3a have similar although not identical effects on MDM2 as well as on

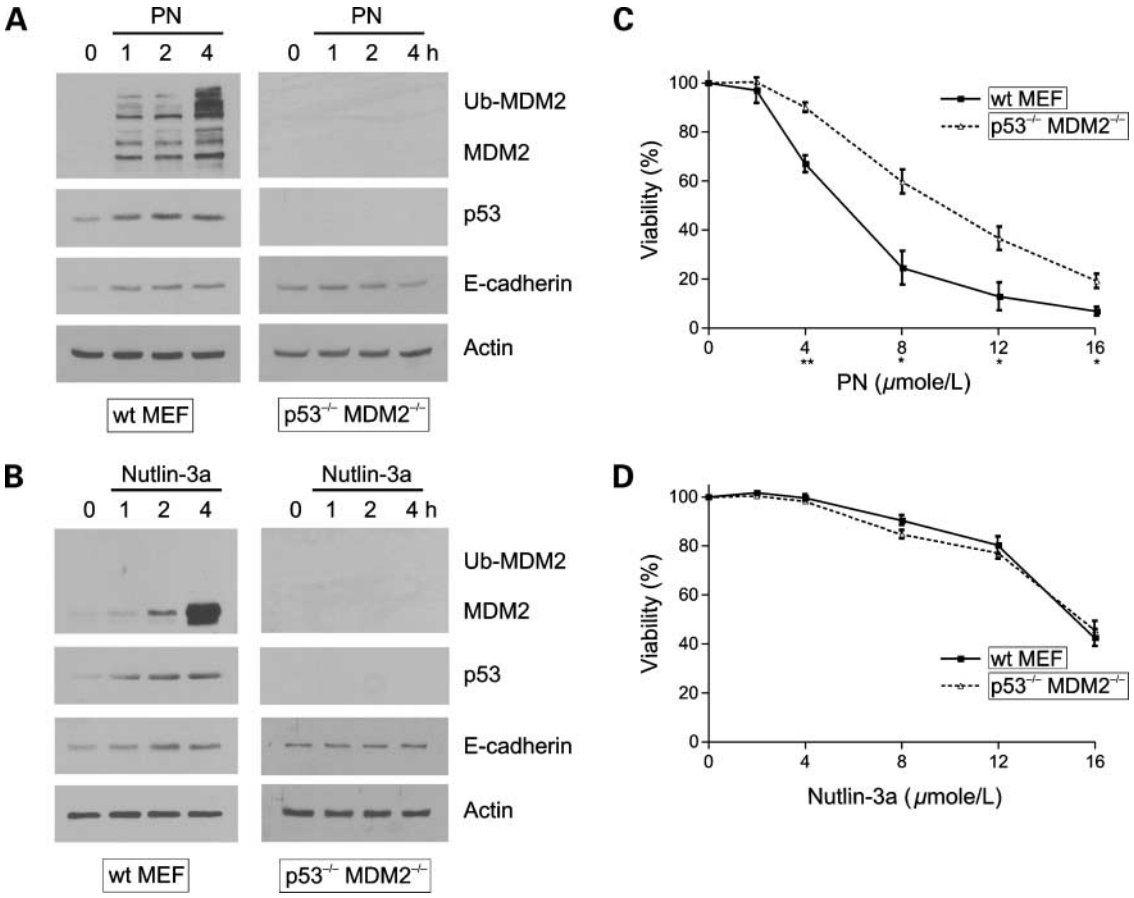

Figure 6. Comparison of PN and nutlin-3a effects on MDM2, p53, and E-cadherin proteins and wild-type and $p53^{-/-}$ $MDM2^{-/-}$ MEF proliferation. **A,** Western blots of whole-cell extract proteins from wild-type and $p53^{-/-}$ $MDM2^{-/-}$ MEFs treated with 15 μmol/L PN for the durations indicated. **B,** Western blots done as described in **A** but following treatment with 10 μmol/L nutlin-3a. **C,** plots of WST-1 viability assays of wild-type (*solid line*, ■) and $p53^{-/-}$ $MDM2^{-/-}$ MEF (*dotted line*, △) cells treated for 48 h with the PN concentrations indicated. Statistical significance of differences in viability between the two cell types following PN treatment is indicated as * ($P < 0.05$) and ** ($P < 0.01$) for individual PN concentrations. **D,** viability plots as described in **C** but following treatment with the nutlin-3a concentrations indicated.

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resulting p53-dependent and p53-independent processes. To better ascertain the cellular consequences of these compounds, wild-type and $p53^{-/-}$ $MDM2^{-/-}$ MEFs were treated with increasing concentrations of PN or nutlin-3a for 48 hours and their viability was determined. We found that cells lacking both p53 and MDM2 exhibited increased resistance to PN compared with their wild-type counterparts (Fig. 6C). Using a two-tailed, equal variance *t* test, we determined that except at the lowest concentration of PN (2 μmol/L), the sensitivity difference between the two cell lines to increasing PN concentrations was statistically significant ($P < 0.005$ for 4 μmol/L PN and $P < 0.05$ for 8, 12, and 16 μmol/L PN). By comparison, nutlin-3a was far less effective than PN in reducing the viability of either wild-type or $p53^{-/-}$ $MDM2^{-/-}$ MEFs (Fig. 6D) and no significant difference was observed in the sensitivity of either wild-type or mutant MEFs. Such would suggest that in this cell type, nutlin-3a was not completely dependent on MDM2 and p53 for its observed toxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Parthenolide promotes the ubiquitination of MDM2 and activates p53 cellular functions

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