

Inhibitors of Ubiquitin-Activating Enzyme (E1), a New Class of Potential Cancer Therapeutics

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Abstract

The conjugation of proteins with ubiquitin plays numerous regulatory roles through both proteasomal-dependent and nonproteasomal-dependent functions. Alterations in ubiquitylation are observed in a wide range of pathologic conditions, including numerous malignancies. For this reason, there is great interest in targeting the ubiquitin-proteasome system in cancer. Several classes of proteasome inhibitors, which block degradation of ubiquitylated proteins, are widely used in research, and one, Bortezomib, is now in clinical use. Despite the well-defined and central role of the ubiquitin-activating enzyme (E1), no cell permeable inhibitors of E1 have been identified. Such inhibitors should, in principle, block all functions of ubiquitylation. We now report 4[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester (PYR-41) as the first such inhibitor. Unexpectedly, in addition to blocking ubiquitylation, PYR-41 increased total sumoylation in cells. The molecular basis for this is unknown; however, increased sumoylation was also observed in cells harboring temperature-sensitive E1. Functionally, PYR-41 attenuates cytokine-mediated nuclear factor- κ B activation. This correlates with inhibition of nonproteasomal (Lys-63) ubiquitylation of TRAF6, which is essential to κ B kinase activation. PYR-41 also prevents the downstream ubiquitylation and proteasomal degradation of κ B α . Furthermore, PYR-41 inhibits degradation of p53 and activates the transcriptional activity of this tumor suppressor. Consistent with this, it differentially kills transformed p53-expressing cells. Thus, PYR-41 and related pyrazones provide proof of principle for the capacity to differentially kill transformed cells, suggesting the potential for E1 inhibitors as therapeutics in cancer. These inhibitors can also be valuable tools for studying ubiquitylation. [Cancer Res 2007;67(19):9472–81]

Introduction

Ubiquitylation is catalyzed by the sequential action of ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin protein ligase (E3). In humans, there is a single essential

E1 and over 30 distinct E2s. E3s, of which there are estimated to be between 500 and 1000, are largely responsible for conferring specificity to ubiquitylation (1, 2). Ubiquitylation is essential to numerous cellular and developmental processes, including, but not limited to, protein quality control, growth, apoptosis, antigen presentation, DNA repair, and signal transduction. Proteins can be modified either with chains of ubiquitin molecules (polyubiquitylation or multiubiquitylation) or by one or more single ubiquitin moieties (monoubiquitylation). The most well-characterized role for ubiquitin is in targeting proteins for degradation by the 26S proteasome after modification with chains of four or more ubiquitins linked through Lys-48 (K48) of ubiquitin. K63-linked polyubiquitin chains play essential roles in the activation of nuclear factor- κ B (NF- κ B) induced by cytokines and engagement of Toll-like receptors (3). K63 ubiquitylation and monoubiquitylation are also both implicated in vesicular trafficking of proteins, nucleocytoplasmic transport, and DNA repair (4, 5). There is also evidence that other ubiquitin linkages play critical roles, including the activation of ubiquitin ligases themselves (6).

Alterations in ubiquitylation are observed in most, if not all, cancer cells. This is manifested by destabilization of tumor suppressors, such as p53, and overexpression of protooncogenes, including c-Myc and c-Jun (1). The growing appreciation of the importance of ubiquitylation has resulted in great interest in targeting components of the ubiquitin-proteasome system in cancer. E3s represent particularly attractive targets because of their role in substrate recognition. However, because of structural similarities that characterize members of each class of E3s, valid concerns have been raised about the feasibility of blocking the function of specific E3s (7). There is, however, some evidence supporting such a strategy, at least for Hdm2/Mdm2, which targets p53 for degradation (8–11).

Although inhibition of the proteasome is considered a relatively nonspecific way to target ubiquitin-dependent protein degradation, the proteasome inhibitor Bortezomib is now being used to treat multiple myeloma and is under evaluation for mantle cell lymphoma and other non-Hodgkin's lymphomas (12, 13). Thus, inhibition of the ubiquitin system at points where there is, at face value, little substrate specificity has the potential to result in a substantial therapeutic index in cancer.

Whereas the proteasome represents the final destination for many ubiquitylated proteins, E1 is the common first step in ubiquitylation, whether or not the modified protein is ultimately degraded by the proteasome. We now report the first cell permeable inhibitor of the ubiquitin E1, 4[4-(5-nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoic acid ethyl ester. We named this pyrazone derivative PYR-41. Among the cellular consequences of exposure of cells to PYR-41 is inhibition of NF- κ B

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-07-0568

activation and increased levels and activity of p53. These findings correlate with the ability of PYR-41 to induce apoptosis in transformed cells, particularly those expressing wild-type p53.

Materials and Methods

Compounds. The pyrazones reported in this study were identified from a commercial screening library purchased from ChemBridge, Inc. Hits were validated based on compounds resupplied from Asinex USA, Ltd., and were further qualified for purity and identity by standard chemical methods.

Plasmids. Plasmids encoding the NF- κ B reporter pNF3TKLuc (14), pGEX-2TK-Ub (15), cyclin E (16), GFP^u (17), pGEX-Nedd4 (11), p53 (18), and E6 (18) have been described. The plasmid encoding glutathione S-transferase (GST)-UbcH5B was generated by subcloning of UbcH5B from pGEM7 into pGEX-KG from Nco I to Sac I.

Reagents and antibodies. Recombinant mouse His₆-E1 was produced in insect cells using constructs provided by Dr. Kazuhiro Iwai (Osaka City University, Osaka, Japan). Purified rabbit E1 was purchased from Boston Biochem. Antibodies against E1 (19) and SUMO-1 (20) were gifts from Drs. Allen Taylor and Mary Dasso, respectively. Recombinant UbcH5B was produced as described (21). GST-ubiquitin in pGEX-2TK and GST-UbcH5B were expressed as described (15, 22). ³²P labeling, GST cleavage, and purification of ubiquitin has been described (22). Recombinant epidermal growth factor (EGF) and tumor necrosis factor- α (TNF- α) were from R&D Systems. Dexamethasone was from Sigma. Human IL-1 α was from PeprroTech. TALON cobalt affinity resin was from Clontech. Protein G Sepharose and glutathione Sepharose were from Amersham Biosciences. Antibodies recognizing EGF receptor (EGFR), phosphorylated EGFR, and Cull1 were from Cell Signaling. Antibodies recognizing Hdm2 (Ab-1 and Ab-2) were from Oncogene. Antibodies recognizing I κ B α , phosphorylated I κ B α , cyclin D3, p53, GST, TRAF6, and GFP were from Santa Cruz Biotechnology. Anti-caspase-9 was from Stressgen.

Cell lines. Jurkat T cells, 2B4 T hybridoma cells, L929, ts20, and tsA1S9 cells (23–25) were cultured in RPMI 1640 supplemented with 10% FCS and 50 μ mol/L of 2-mercaptoethanol. HEK293 Tet-On cells expressing myc-SUMO (26), HeLa, A9, C8 (27), U2OS, and U2OS-pG13 (11) were cultured in DMEM supplemented with 10% FCS. Retinal pigment epithelial (RPE) and RPE-E1A cells were maintained as described (11). All media was supplemented with 100 units/mL of penicillin and 100 μ g/mL of streptomycin.

In vitro ubiquitylation reactions. Rabbit or mouse E1 (~250 ng) was incubated with ³²P-ubiquitin (28) in 1 \times reaction buffer [50 mmol/L Tris (pH 7.4), 0.2 mmol/L ATP, 0.5 mmol/L MgCl₂] at room temperature for 15 min. In some experiments, the His-tagged mouse E1 was bound to TALON cobalt affinity resin before carrying out incubations and reactions. For E2 assays, GST-UbcH5B (~900 ng) was bound to glutathione Sepharose beads. Mouse E1 and ³²P-ubiquitin were added to the beads in 1 \times reaction buffer and incubated as for E1 reactions. Samples were heated in nonreducing SDS-PAGE sample buffer and resolved by SDS-PAGE. Thioesters with ubiquitin were visualized by Storm PhosphoImager (Amersham). *In vitro* autoubiquitylation reactions were carried out on E3 prebound to anti-GST on protein G beads essentially as described (22).

Immunoprecipitation and immunoblotting. Except where noted, cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μ g/mL aprotinin, 100 μ g/mL phenylmethylsulfonyl fluoride, and 5 μ g/mL leupeptin] and insoluble material was pelleted by centrifugation at 15,000 \times g for 20 min. For immunoprecipitation, the supernatant was diluted 4-fold with PBS and incubated with protein G-bound specific antibody for 3 to 5 h at 4°C. Samples were heated in SDS-PAGE sample buffer containing reducing agent (DTT), resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated with 5% bovine serum albumin (BSA) in TBST [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.05% Tween 20] before addition of specific antibody. After thorough washing, bound antibodies were detected with horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence (Amersham). For analysis of cellular E1 thioesters with ubiquitin, RPE

cells were treated as indicated and processed using a urea-containing buffer for cell lysis as described (19), before resolution by SDS-PAGE.

In vitro degradation assay. Cyclin E degradation assays were carried out using S100 cytosol prepared from CA46 cells (16). Human cyclin E was synthesized by coupled transcription/translation according to the manufacturer's instructions (Promega) using ³⁵S-labeled Met and Cys. For each reaction, 50 μ g of S100 were first incubated with DMSO or indicated compounds in a 50- μ L reaction mixture for 30 min at room temperature. Labeled cyclin E was then added, and the cytosolic preparation was further incubated up to 90 min. For some reactions, 250 ng of recombinant E1 were added to the reaction mixture before the addition of cyclin E. Aliquots of 10 μ L were taken at the indicated times. After boiling in sample buffer, they were resolved by SDS-PAGE, followed by autoradiography. p53 degradation was carried out using wild-type p53 and human papillomavirus-16 E6 that was translated *in vitro* as described for cyclin E. Degradation of p53 was allowed to proceed by mixing rabbit reticulocyte lysates containing p53 with either control lysate or lysate containing E6 in a 50- μ L reaction, containing 25 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 3 mmol/L DTT, for up to 3 h at room temperature. Where appropriate, p53 was preincubated with PYR-41 or DMSO for 15 min before adding E6. Aliquots were removed at the indicated times and analyzed by SDS-PAGE followed by autoradiography.

Luciferase assay. Assays were carried out using the Luciferase Assay System (Promega) according to manufacturer's instructions.

Results

PYR-41 inhibits the ubiquitin E1 but not E2. We previously described a series of 5-deazaflavins (HL198s), identified through an *in vitro* high-throughput screen, as inhibitors of the ubiquitin ligase activity of Hdm2 that stabilize and activate p53 (11). In an attempt to identify more potent inhibitors, a larger library was screened. Many of the "hits" prevented ubiquitylation mediated by multiple E3s of different structural classes (data not shown). Because of the nature of the screen, inhibitors of either E1 or E2, which both contain active site cysteines that form thioester intermediates with ubiquitin (Ub~E1/Ub~E2) and whose inactivation would readout as inhibition of multiple E3s, would also score as hits. Therefore, these small molecules were tested for inhibition of ubiquitin thioester bond formation with E1, as well as with the E2 used in the high-throughput screen (Ube2d2/UbcH5B). Some compounds inhibited both E1 and E2 (data not shown), suggesting that they might react indiscriminately with free thiols. However, one compound, PYR-41, showed selective inhibition of E1. It blocked loading of immobilized His₆-tagged E1 with ubiquitin with an IC₅₀ of <10 μ mol/L (>60% inhibition at 10 μ mol/L; Fig. 1A). At 50 μ mol/L, there was at least 95% inhibition. In general >90% inhibition at 50 μ mol/L was observed within 3 to 5 min of treatment (data not shown). Inhibition of E1 activity persisted even when immobilized E1 was washed thoroughly before the thioester assay (Fig. 1A). This raises the possibility that this compound might covalently modify E1. PYR-41 did not affect the transfer of ubiquitin to E2 from E1 that was preloaded with ubiquitin (not shown). Similarly, no inhibition was observed when immobilized UbcH5B was treated with PYR-41 and then extensively washed before addition of E1 (Fig. 1B compare lanes 1 and 2). Similar results were obtained with His₆-tagged UbcH5B and with another E2, Ube2G2 (data not shown). These results show that PYR-41 directly inhibits E1, but not E2.

The structure of PYR-41 suggests that it could be a target for nucleophilic attack (Fig. 1C) and potentially could react with the active site cysteine of E1. This could occur either by Michael addition to the exocyclic double bond (Fig. 1C-b) or via reaction with the *N*-aryl bond (Fig. 1C-a). To evaluate whether other related

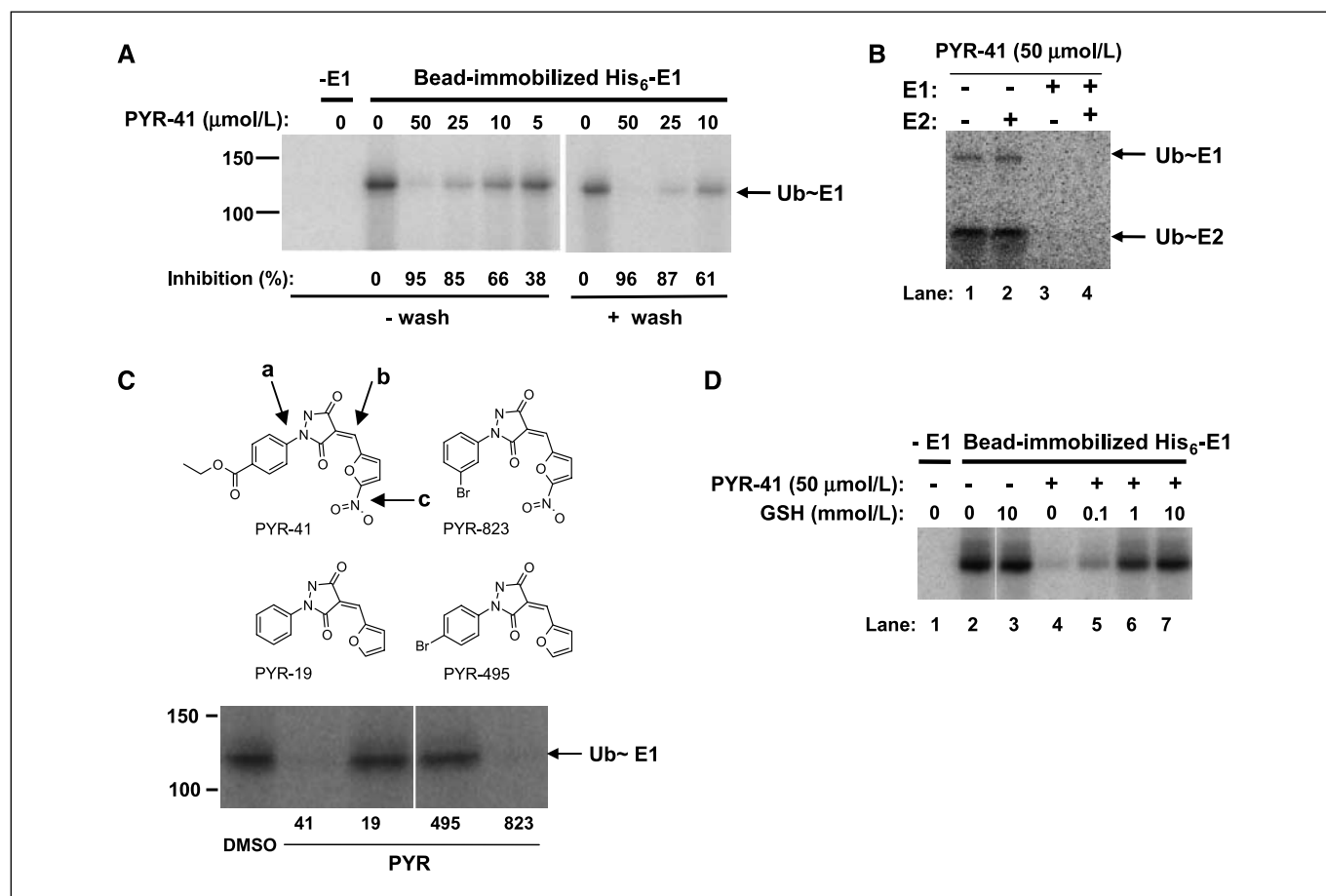


Figure 1. PYR-41 inhibits the ubiquitin E1. **A**, His₆-tagged mouse E1 was immobilized on TALON beads and treated with PYR-41 for 15 min, as indicated. Beads were either washed thoroughly in 50 mmol/L Tris-HCl (pH 7.4) containing 0.05% Triton X-100 to remove free PYR-41 or not washed, as indicated. Formation of Ub~E1 thioester bonds was assessed using ³²P-labeled ubiquitin and resolution of samples by SDS-PAGE under nonreducing conditions. *Bottom*, percentage inhibition for treated samples. **B**, GST-UbcH5B (E2) bound to glutathione Sepharose beads was incubated with purified soluble E1 in reaction buffer. Formation of thioester linkages of ³²P-ubiquitin with E2 and E1 was assessed. *Lane 2*, immobilized GST-UbcH5B was pretreated with PYR-41 (50 $\mu\text{mol/L}$) for 15 min before washing the beads and adding E1; *lane 3*, E1 was pretreated with PYR-41 for 15 min before addition to E2; *lane 4*, both E1 and immobilized E2 were exposed to PYR-41 before assessment of thioester bond formation. **C**, chemical structures of PYR-41 and related pyrazones; N-aryl bond (*a*), exocyclic double bond (*b*), and nitro group (*c*). *Bottom*, rabbit E1 (500 ng) was incubated with indicated compounds (50 $\mu\text{mol/L}$) for 15 min at 37°C. ³²P-labeled ubiquitin and reaction buffer were then added to carry out thioester assays. Samples were resolved by nonreducing SDS-PAGE. **D**, 2 pmol of E1 immobilized as in **A** were pretreated with PYR-41 or DMSO, together with GSH, as indicated in 30 μL for 15 min. Beads were washed, and formation of Ub~E1 thioester bonds was then assessed as in **A**.

pyrazones would have similar activity, three related pyrazones were evaluated. One of these, PYR-823, similarly inhibited loading of E1 with ubiquitin but did not directly affect E2 activity (Fig. 1C and data not shown), whereas the other two had no effect at 50 $\mu\text{mol/L}$. Based on this limited analysis, it seems that the nitro substitute on the furan ring may be important for activity (Fig. 1C-c), whereas the carboxyethyl ester substitute of the benzene ring of PYR-41 is less important. These substitutions may play a role in allowing access to the site of interaction on E1 or may control reactivity of these pyrazones. To assess potential reactivity of PYR-41 with sulfhydryls, immobilized E1 was incubated with PYR-41 in the presence of an excess of reduced glutathione (GSH) as a source of reactive thiols. After extensive washing, the capacity to form Ub~E1 thioesters was assessed. GSH totally abolished inhibition of E1 activity by PYR-41 when it was added at 10⁴-fold to 10⁵-fold excess relative to E1 (Fig. 1D, lanes 6 and 7). This supports the notion that PYR-41 may function by covalently modifying E1, perhaps on its active site cysteine.

The specificity of PYR-41 was further evaluated by assessing effects on caspase activity in cytosol from 2B4 T-cell hybridoma

cells induced to undergo apoptosis. No inhibition of caspase activity by PYR-41 was observed in cell lysates from apoptotic cells. In fact, treatment of 2B4 cells with PYR-41 activates caspases and induces apoptosis (Supplementary Fig. S1 and data not shown). The effects of PYR-41 on purified Nedd4 were also evaluated. Partial inhibition of autoubiquitylation of this HECT domain E3 was observed at a concentration of PYR-41 that almost completely inhibited Ub~E1 thioesters (Supplementary Fig. S2). Similar results were obtained with another HECT E3, E6-AP. Thus, in the setting of the purified protein, PYR-41 seems to have some activity toward HECT E3s, but substantially less than that toward E1.

PYR-41 acts on E1 to block protein degradation. To further characterize the inhibition of E1 by PYR-41, the degradation of cyclin E was assessed using cytosol (S100) that contains all the components of the ubiquitin-proteasome system necessary to degrade this protein (16, 18). PYR-41 efficiently blocked cyclin E degradation (Fig. 2A, top), which was reversed by addition of exogenous E1 at a final concentration of 50 nmol/L after a period of 30 min (bottom). In contrast, inhibition of cyclin E degradation by proteasome inhibitor (MG132) was not overcome by addition of

E1. PYR-41 also blocked the *in vitro* ubiquitin-mediated degradation of p53 in rabbit reticulocyte lysates when stimulated by human papilloma virus E6 (Fig. 2B; ref. 28). Thus, PYR-41 inhibits E1 to prevent ubiquitin-mediated proteasomal degradation.

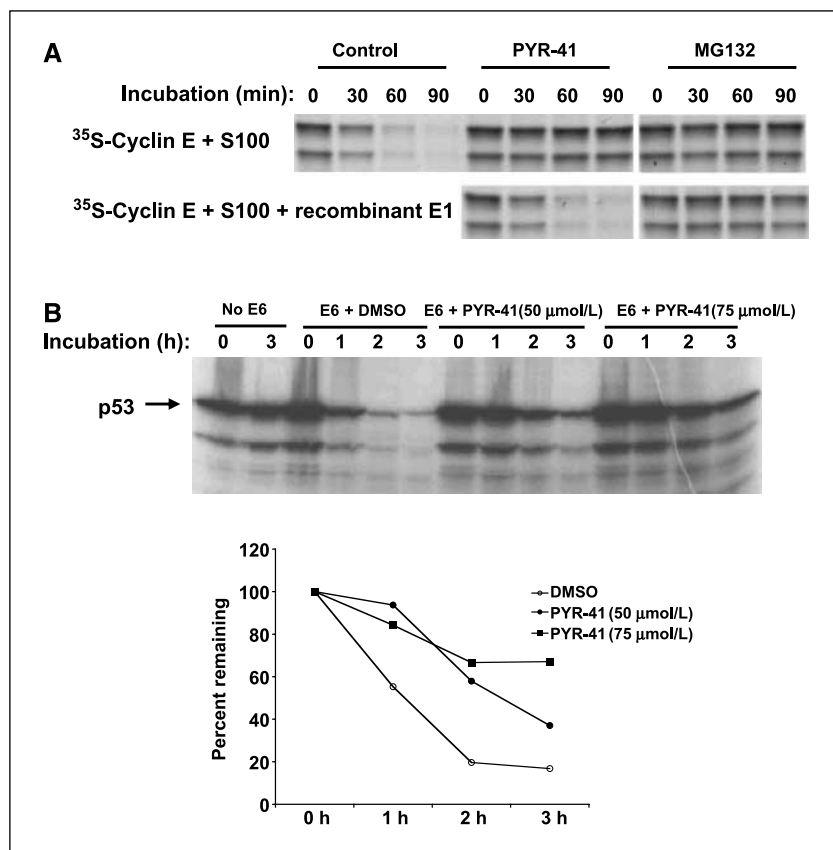
PYR-41 decreases the level of E1 ~ Ub thioesters in cells and prevents proteasome inhibitor-induced accumulation of ubiquitylated proteins. To determine if PYR-41 is active in cells, its effect on the steady-state level of cellular Ub ~ E1 thioesters was evaluated (19). PYR-41 markedly reduced Ub ~ E1 thioesters, whereas the Hdm2 inhibitor HLI98C was without effect (Fig. 3A). Results similar to those with PYR-41 were obtained with PYR-823 (not shown). For PYR-41, the IC₅₀ for inhibition of thioester formation in cells was between 10 and 25 μmol/L (data not shown). To assess the effects of PYR-41 on the accumulation of ubiquitin conjugates, cells were treated with PYR-41 in the presence or absence of a proteasome inhibitor (ALLN), which is known to increase total cellular ubiquitylation (29). Consistent with its proposed function, PYR-41 blocked accumulation of ubiquitin conjugates in response to the proteasome inhibitor ALLN over a period of 90 min (Fig. 3B, compare lanes 3 and 4). Notably and unexpectedly, we generally did not observe a decrease in total ubiquitin conjugates with PYR-41 in the absence of proteasome inhibitor treatment (Fig. 3B and C, right). In some cases, we observe an increase in high molecular weight ubiquitylated species with a corresponding decrease in those at the lower molecular weight region with no net change in total ubiquitin immunoreactivity (Fig. 3B, compare lanes 1 and 2).

Inhibition of E1 increases sumoylation in cells. NEDD8 and SUMO-1 are conjugated to proteins on primary amines in a manner that involves an enzyme cascade similar to that for ubiquitin (30).

As a control for ubiquitylation, we evaluated neddylation and sumoylation in cells treated with PYR-41. No change in total neddylation was observed (not shown). Similarly, no change in neddylation of the well-characterized substrate Cul-1, which undergoes continuous cycles of neddylation and deneddylation in the context of SCF ubiquitin ligases (31), was seen for up to 8 h (Supplementary Fig. S3). In contrast, an unexpected rapid increase in total sumoylated proteins was observed (Fig. 3C, left; Supplementary Fig. S4). To determine if loss of ubiquitin E1 activity can account for this increased sumoylation, we examined two cell lines expressing temperature-sensitive E1 (tsE1; ref. 24). For both of these, incubation at the restrictive temperature significantly increased sumoylation, which was particularly noticeable after 24 h (Fig. 3D). No increase in sumoylation of L929, the parental cell of tsA1S9, was observed (Supplementary Fig. S5). Thus, although other unknown effects could contribute to increased sumoylation with PYR-41, based on two different tsE1 cell lines, inhibition of the ubiquitin E1 is sufficient to result in an increase in total sumoylation. For a few proteins, it has been shown that SUMO-1 and ubiquitin can compete for the same lysines (32). The striking increase in sumoylation suggests that either this competition is more general than appreciated or that there are other cross-regulatory mechanisms involving these two protein modifications yet to be uncovered. Dissecting out this relationship and how this relates to the reported increase in sumoylation in HepG2 cells upon treatment with proteasome inhibitor (33) will require further study.

PYR-41 inhibits both proteasome-dependent and proteasome-independent activities of ubiquitylation. An inhibitor of the ubiquitin E1 should block both proteasomal and nonproteasomal functions of ubiquitylation. Accordingly, PYR-41 blocked both the

Figure 2. PYR-41 inhibits ubiquitin-mediated proteasomal degradation *in vitro*. **A**, ³⁵S-labeled *in vitro* translated cyclin E (which migrates as two bands due to alternative initiation) was added to the S100 fraction, and its degradation in the presence or absence of PYR-41 (50 μmol/L) or MG132 (50 μmol/L) was assessed. **Bottom**, 30 min after the cytosol was treated with PYR-41, 250 ng of recombinant mouse E1 (final concentration, 50 nmol/L) was added to the mixture, together with *in vitro* translated cyclin E. **B**, ³⁵S-labeled p53 and E6 were incubated in rabbit reticulocyte lysates in the presence or absence of PYR-41, as indicated, and E6-dependent degradation of p53 was assessed. Loss of p53 is quantified in the accompanying graph.



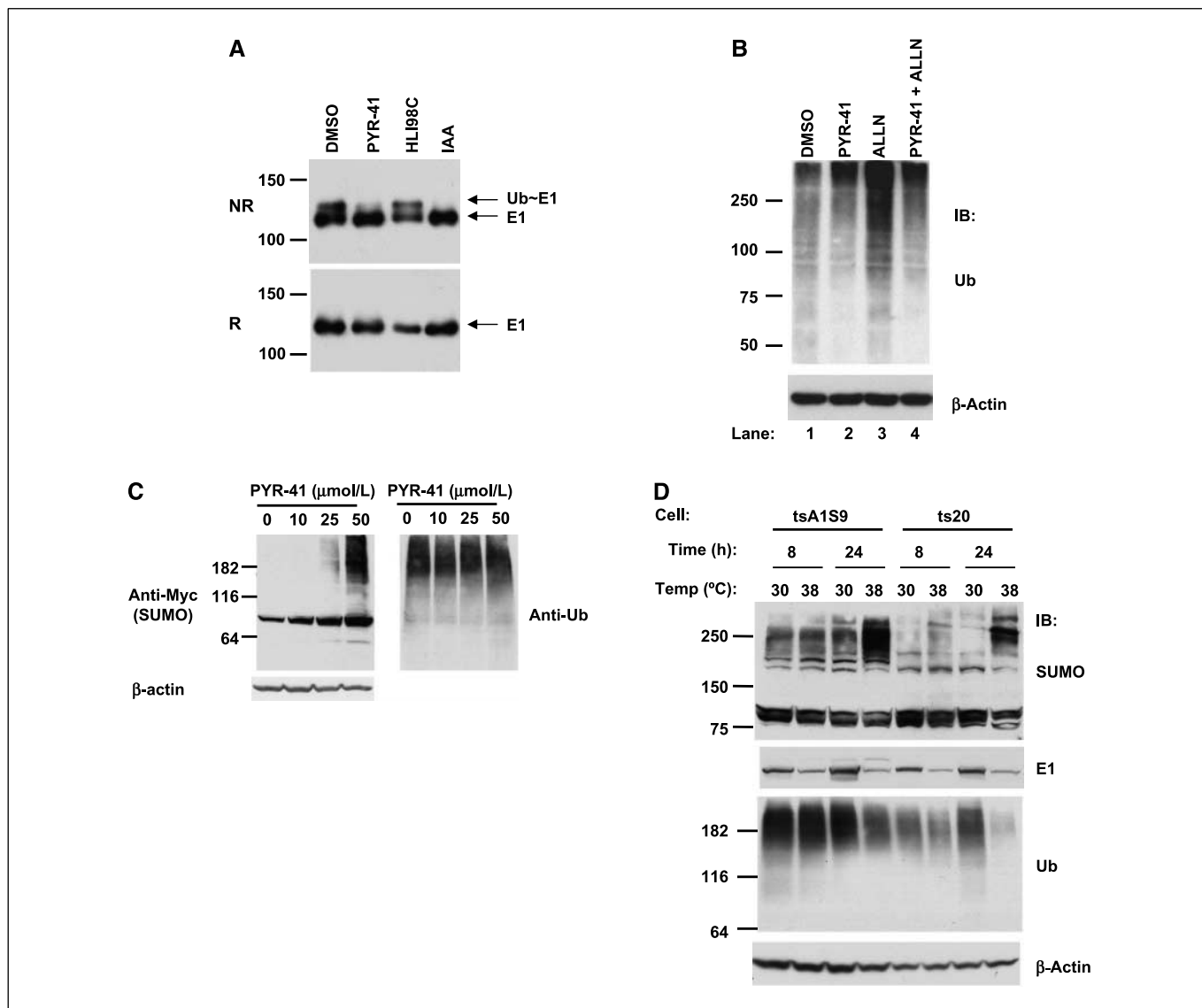


Figure 3. PYR-41 is active in cells and enhances accumulation of sumoylated proteins. *A*, RPE cells were treated with the indicated compounds [50 μ mol/L PYR-41, 20 μ mol/L HLI98C, or 10 mmol/L of the nonspecific alkylating agent iodoacetamide (*IAA*)] for 30 min. Cell lysates were heated in either nonreducing (*NR*) or reducing (*R*) SDS-PAGE sample buffer, followed by SDS-PAGE and immunoblotting with anti-E1. *B*, RPE cells were exposed to PYR-41 (50 μ mol/L) or DMSO for 30 min at 37 $^{\circ}$ C before treatment with or without the proteasome inhibitor ALLN (50 μ mol/L) for an additional 90 min at 37 $^{\circ}$ C. Total ubiquitination was assessed by immunoblotting with anti-ubiquitin. *C*, HEK293 stably expressing tetracycline-inducible Myc-SUMO-1 were incubated with doxycycline (2 μ g/mL) for 20 h before incubation with PYR-41 for 4 h at the indicated concentrations, followed by immunoblotting. A β -actin blot is shown as a loading control. *D*, cells expressing tsE1 SUMO-1 and also evaluated for E1 and β -actin.

ubiquitylation and proteasomal degradation of a test substrate, GFP¹¹, which has been used to assess the function of the ubiquitin-proteasome system (Fig. 4A; ref. 34) and also results in an increased level of cyclin D3 (Fig. 4B). Among the nonproteasomal roles of ubiquitylation is down-regulation of signaling by EGFR, which undergoes ligand-induced tyrosine phosphorylation. This stimulated phosphorylation leads to EGFR ubiquitylation and eventual trafficking to and destruction of activated receptors in lysosomes. This process is dependent on monoubiquitylation and K63-mediated ubiquitylation of EGFR, as well as monoubiquitylation of multiple other proteins involved in membrane trafficking (35). Consistent with its predicted activity, ligand-induced EGFR ubiquitylation was decreased by PYR-41, whereas EGFR phosphorylation was increased in intensity (Fig. 4C). As shown in Fig. 4D, this enhanced

phosphorylation persists for at least 30 min, and in this experiment, where EGFR down-regulation was easily observed, loss of EGFR was significantly delayed by PYR-41. Thus, PYR-41 inhibits ligand-induced ubiquitylation of EGFR, prolongs the time during which receptors are activated, and can attenuate ligand-mediated receptor down-regulation.

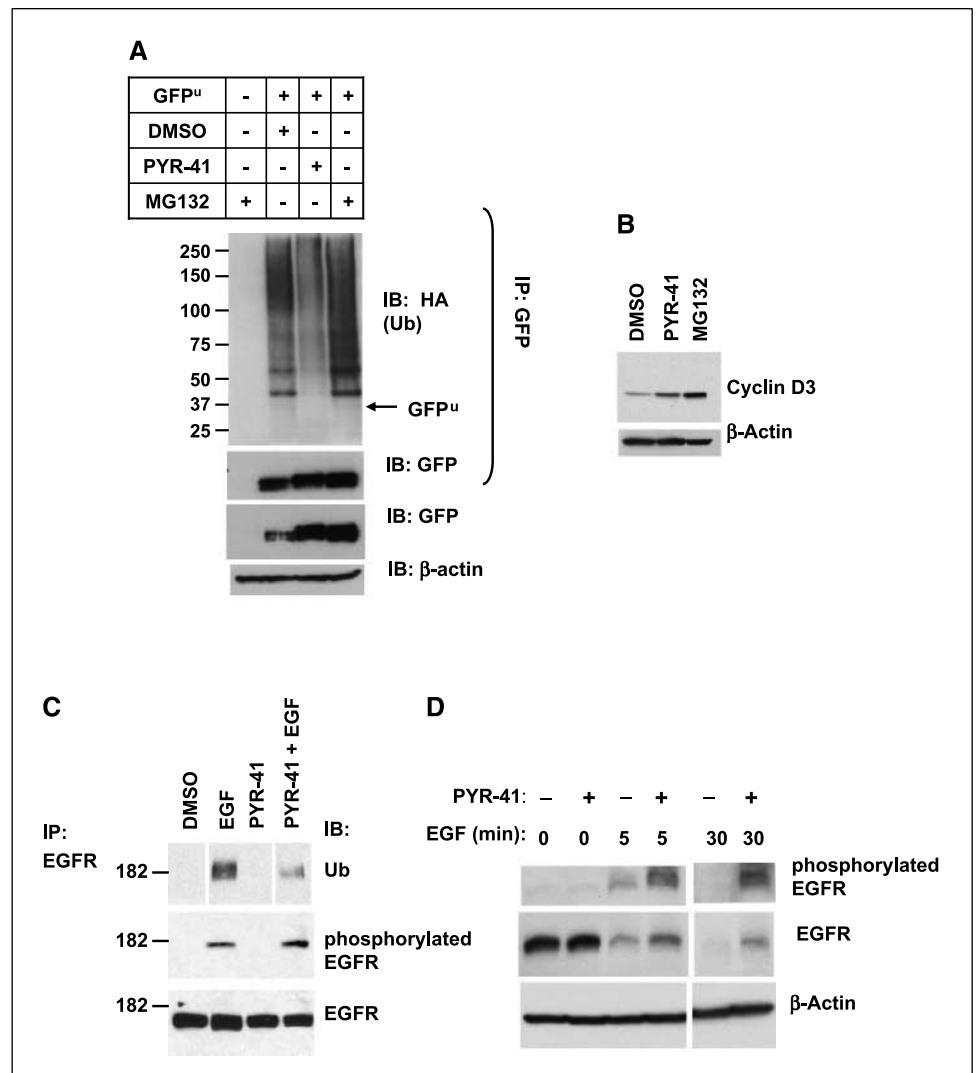
The E1 inhibitor blocks cytokine-induced activation of NF- κ B. Activation of the NF- κ B family of transcription factors is critical to inflammatory responses and is implicated in tumorigenesis (36). The efficacy of proteasome inhibitors in cancer has been attributed, at least in part, to blockade of NF- κ B activation by preventing ubiquitin-mediated proteasomal degradation of the NF- κ B inhibitor I κ B α (37). IL-1 α activates NF- κ B through a pathway involving modification of TRAF6 with

K63-linked ubiquitin chains, which do not target TRAF6 for proteasomal degradation (3). This ubiquitylation leads to activation of I κ B kinase (IKK), which phosphorylates I κ B α , which is then ubiquitylated and degraded by the proteasome. As shown (Fig. 5A), PYR-41 markedly inhibited transactivation of an NF- κ B-driven luciferase reporter when assessed after 2 h of treatment with IL-1 α , with >60% inhibition at 1 ng/mL of IL-1 α . When the signaling pathway from IL-1 α leading to NF- κ B activation was examined after stimulation at 10 ng/mL of IL-1 α , PYR-41 was found to largely prevent TRAF6 ubiquitylation (Fig. 5B). Consistent with inhibition of IKK activation, PYR-41 also delayed the phosphorylation of I κ B α (Fig. 5C, middle). Thus, PYR-41 inhibits both biochemical activation of NF- κ B and has substantial effects on signaling mediated through this transcription factor. Additionally, no net decrease in I κ B α was observed in cells treated with PYR-41 over a period of 5 min (Fig. 5C, top). When NF- κ B was stimulated through TNF- α , degradation of I κ B α was similarly prevented (Fig. 5D). These results indicate that, unlike proteasome inhibitors, PYR-41 can reduce NF- κ B activation by inhibiting ubiquitylation of upstream signaling molecules and by blocking the more downstream proteasomal degradation of I κ B α .

PYR-41 accumulates and activates p53 in cells. p53 is a tumor suppressor whose level and activity are tightly controlled by Hdm2-dependent, ubiquitin-mediated proteasomal degradation. We predicted that PYR-41 should inhibit Hdm2, albeit indirectly, and thereby stabilizes p53 and might possibly increase its activity. To evaluate the effect of E1 inhibition on p53 activity, a cell line that expresses wild-type p53 and also stably expresses a luciferase reporter under the control of a p53 response element was used (U2OS-pG13; ref. 11). PYR-41 at 20 μ mol/L induced p53 reporter activity to a level comparable with an optimal dose of Adriamycin, a chemotherapeutic agent known to activate p53 (Fig. 6A). PYR-41 increased the level of p53 and also led to increased Hdm2, which is known to undergo autoubiquitylation and proteasomal degradation; moreover, it led to a substantial increase in the cellular level of the p53 responsive cell cycle inhibitor p21 (WAF-1), which is induced by activation of p53 (Fig. 6B; ref. 38).

PYR-41 preferentially kills transformed cells with wild-type p53. Transformed cells are particularly prone to undergo apoptosis in response to activation of p53 (39, 40). To evaluate whether PYR-41 induces differential killing of transformed cells expressing wild-type p53, RPE cells and E1A-transformed RPE cells (11) were compared. E1A-transformed cells were killed within 20 h of

Figure 4. PYR-41 inhibits substrate ubiquitylation. **A**, U2OS cells were transfected with GFP^u, which includes GFP and a known targeting signal for ubiquitylation and proteasomal degradation. At 36 h after transfection, cells were treated for 8 h with DMSO, PYR-41 (50 μ mol/L), or MG132 (50 μ mol/L). In the top two blots, cell lysates were immunoprecipitated with anti-GFP, followed by immunoblotting, as indicated. The position of GFP^u in the anti-HA blot is indicated; in the bottom two blots, immunoblotting of equal amounts of lysate from each transfection was carried out. **B**, HeLa cells were treated with MG132 (50 μ mol/L) or with PYR-41 (50 μ mol/L) for 6 h before lysis and immunoblotting, as indicated. **C**, HeLa cells were treated with EGF (100 ng/mL) for 5 min with or without pretreatment with PYR-41 for 15 min and assessed for EGFR levels, EGFR phosphorylation (using anti-phosphorylated EGFR), or immunoprecipitated with anti-EGFR and assessed for EGFR ubiquitylation. **D**, HeLa cells were stimulated with EGF (100 ng/mL), as indicated, with or without pretreatment with PYR-41 for 15 min and immunoblotted, as indicated.



treatment with PYR-41 in a dose-dependent manner, whereas untransformed RPE cells were relatively resistant (Fig. 6C). The capacity of PYR-41 to cause cell death was not reversed by washing of cells to remove this reagent after a 2-h incubation (Supplementary Fig. S6). The apoptotic nature of this cell death in transformed RPE cells was confirmed by poly (ADP-ribose) polymerase cleavage and DNA fragmentation (not shown). Consistent with the essential role of E1 in cell proliferation, some inhibition of cell growth of nontransformed RPE cells is observed at higher concentrations (20 $\mu\text{mol/L}$), whether or not cells were washed extensively after a 2-h incubation (Supplementary Fig. S7). To determine whether death of transformed cells induced by PYR-41 correlates with p53 activity, we examined E1A and Ha-ras transformed mouse embryonic fibroblasts (MEF) from wild-type (C8) and p53-deficient mice (A9; ref. 27). C8 cells exhibited a substantially greater dose-dependent increase in cell death up to 24 h compared with A9 cells (Fig. 6D). However, as the time of incubation increased beyond this, the differences between the two cell lines diminished. Indicative of apoptosis, cleavage of pro-caspase-9 was observed after 8 h treatment with PYR-41 in C8 cells, but not in A9 cells (Supplementary Fig. S8). Thus, p53-expressing transformed cells are relatively more susceptible to apoptotic cell death induced by PYR-41. However, as might be expected for a reagent with pleiotropic effects, p53-negative cells are not immune to the effects of this reagent when examined at longer times after exposure. This likely reflects the roles of ubiquitylation in

regulating numerous cellular processes, including cell cycle and NF- κ B activation.

Discussion

PYR-41 and related pyrazones are the first described reagents that enter cells, inhibit the ubiquitin E1, and differentially kill transformed cells. Thus, they represent the basis for a potential new class of therapeutics, as well as new tools to probe the functions of the ubiquitin system. For example, PYR-41 has brought to light the reciprocal relationship between ubiquitylation and sumoylation, which we confirmed in tsE1 cells and will now stimulate research into the molecular basis for this relationship. We have also shown that, when ubiquitylation is inhibited, EGFR phosphorylation is prolonged and receptor down-regulation is attenuated. Again, these findings should allow for further evaluation of the complexities of the relationship between phosphorylation and ubiquitylation in tyrosine kinase signaling pathways.

One potential limitation of PYR-41 is specificity. Our data indicate that there is no substantial inhibition of E2 or caspases. Furthermore, we find no evidence for inhibition of cellular neddylation, and net cellular sumoylation actually increases, consistent with our findings in tsE1 cells. However, there seems to be partial inhibition of HECT domain E3s *in vitro*, whether PYR-41 actually inhibits these enzymes in cells remains to be

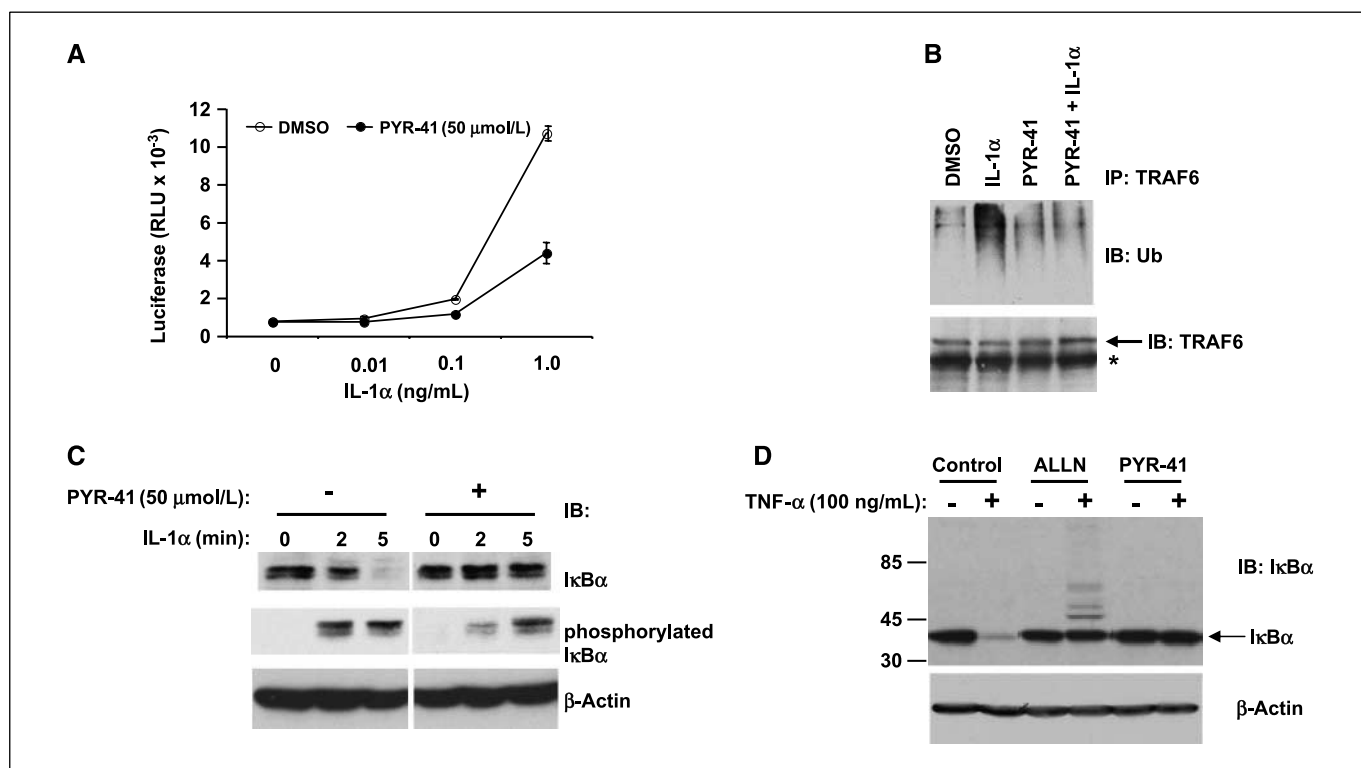


Figure 5. PYR-41 inhibits NF- κ B activation. **A**, HeLa cells were transfected with a luciferase reporter under the control of a NF- κ B response element and treated with IL-1 α for 2 h with or without pretreatment of PYR-41 for 30 min. Lysates were assessed for luciferase activity. Data represents standard deviation of three independent experiments. **B**, HeLa cells were treated with PYR-41 (50 $\mu\text{mol/L}$) for 15 min and then stimulated with IL-1 α (10 ng/mL) for 5 min. Immunoprecipitates were resolved by SDS-PAGE, and membranes were immunoblotted. The asterisk represents heavy chain of immunoglobulin. **C**, HeLa cells were pretreated with PYR-41 for 10 min before IL-1 α stimulation (10 ng/mL) for the indicated times. Cell lysates were immunoblotted with antibodies specific for I κ B α or phosphorylated I κ B α . **D**, 2B4 T-cell hybridoma cells were incubated with DMSO, PYR-41 (50 $\mu\text{mol/L}$), or a peptide aldehyde proteasome inhibitor, ALLN (50 $\mu\text{mol/L}$), for 15 min before treatment with TNF- α where indicated. Note the higher molecular weight forms indicative of I κ B α ubiquitylation that are seen only when TNF- α -induced degradation is inhibited by proteasome inhibitor.

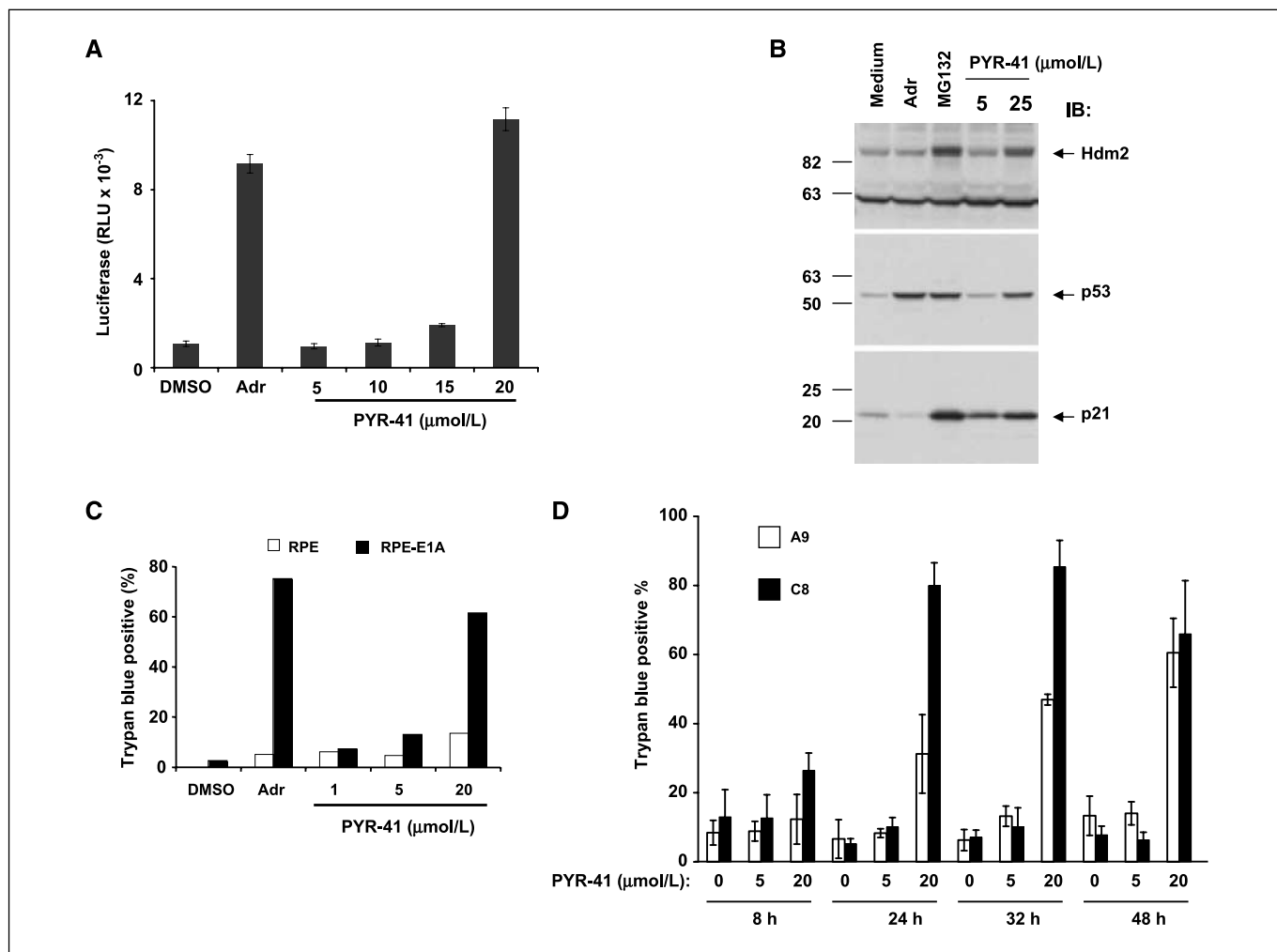


Figure 6. PYR-41 activates p53. *A*, U2OS cells stably transfected with a p53-response element-driven luciferase reporter (U2OS-pG13) were treated with DMSO, 1 μg/mL Adriamycin (*Adr*), or the indicated amounts of PYR-41 for 20 h before assessment of luciferase activity. *B*, U2OS cells were treated with 1 μg/mL Adriamycin, 50 μmol/L MG132, or the indicated amounts of PYR-41 for 6 h before immunoblotting as indicated. *C*, RPE and RPE-E1A cells were incubated for 20 h with the indicated additions before assessment of cell viability by trypan blue exclusion. *D*, transformed MEFs from wild-type (C8) and p53-deficient (A9) mice were treated with PYR-41 (50 μmol/L) as indicated. Cell viability was assessed by trypan blue exclusion.

determined. Regardless, further structure-activity relationships and modeling will be required to generate reagents with greater specificity. Thus, use of this reagent must be carried out with the understanding that it can have other as yet unknown effects.

It will also be of interest, and critical to future structure-activity relationship studies, to conclusively establish the molecular basis on how PYR-41 inhibits E1. Its structure, the apparent irreversible nature of E1 inhibition, and the quenching of activity by a marked excess of free thiols together provide strong circumstantial evidence that PYR-41 functions by blocking the active site cysteine of E1. Molecular modeling based on a partial structure of the ubiquitin E1 (41) is consistent with such a mechanism.⁶ Attempts to definitively establish whether this is the case are ongoing.

A major question that arises from our studies is the potential to use inhibitors of E1 as therapeutics in cancer. There is some

skepticism regarding their potential utility stemming from their predicted effects on multiple proteins and pathways. This is in contrast to the current emphasis on development of highly specific targeted therapeutics (42–45). To date, however, Hdm2 is the only E3 for which specific inhibitors have been reported (8–11), and the general feasibility of inhibiting specific E3s remains an area of debate in the ubiquitin and oncology communities (7). The well-founded emphasis on highly targeted therapeutics notwithstanding, it is now evident that drugs that have more general effects on protein fate can have significant therapeutic indices. Among these are geldanamycin and related ansamycins that inhibit Hsp90 (46, 47). Because of its ability to target EGFR for degradation, 17-AAG, a geldanamycin derivative, is being actively evaluated in breast cancer and other malignancies. Proteasome inhibitors were first developed with the idea of inhibiting muscle wasting and cachexia. With the realization that they could inhibit the activity of the prosurvival transcription factor NF-κB by preventing ubiquitylation and subsequent proteasomal degradation of IκB after its phosphorylation by IKK, proteasome inhibitors have been evaluated for use in the treatment of cancer. Bortezomib, the first of

⁶ Y. Yang, Z. Hu, and A.M. Weissman, unpublished observations.

the proteasome inhibitors in clinical use, is now being used for treatment of multiple myeloma and is under evaluation for other B-cell lymphomas (12).

PYR-41 and related pyrazones also affect the fate and activity of proteins implicated in a number of different cellular processes. As they inhibit both proteasomal and nonproteasomal functions of the ubiquitin system, the range of processes targeted by E1 inhibitors are potentially greater than those affected by proteasome inhibitors. For example, PYR-41 directly affects NF- κ B activation at multiple steps. These include inhibiting formation of K63-linked polyubiquitin chains essential to activation of IKK, as well as by blocking ubiquitin-dependent proteasomal degradation of I κ B, wherein proteasome inhibitors are predicted to act. Thus, one potential advantage of using an E1 inhibitor therapeutically might be in inflammatory processes or malignancies associated with increased activation of NF- κ B.

Most importantly, PYR-41 also has a striking capacity for inhibiting loss of p53 and increasing levels of the p53-induced cell cycle inhibitor p21. Further, we find that PYR-41 differentially targets p53-positive cells for apoptosis. This suggests that even considering the multiple effects of this reagent, the presence of wild-type p53 represents a dominant determinant of susceptibility to cell death. Whether this holds up, as additional model systems are assessed and additional E1 inhibitors are evaluated, will be of great interest.

The identification of PYR-41 and related pyrazones as inhibitors of the ubiquitin E1 represents an important step

forward in developing leads for preclinical evaluation of inhibitors of E1 in cancer and potentially other diseases. PYR-41 increases the level of a cell cycle inhibitor, activates p53, and inhibits NF- κ B activation. All of these are desirable effects for a cancer therapeutic. However, as is the case for proteasome inhibitors, inhibitors of the ubiquitin E1 are also predicted to result in undesirable effects on protein dynamics. Thus, it will be critical to determine, on a case-by-case basis, how E1 inhibitors might be best used when evaluated in preclinical models.

There is clearly much more to be done to determine whether inhibitors of E1 can be efficacious therapeutics and the extent to which their specificity can be optimized. The identification and characterization of PYR-41 as a cell permeable inhibitor of this critical enzyme represent an important first step.

Acknowledgments

Received 2/14/2007; revised 6/15/2007; accepted 7/27/2007.

Grant support: Japanese Society for the Promotion of Science (J. Kitagaki) and Center for Cancer Research, National Cancer Institute.

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We thank Drs. P.B. Chock, M. Dasso, K. Iwai, T. Li, S. Qian, A. Taylor, and J.W. Yewdell for critical reagents, Drs. A. Fotia, M.H. Glickman, K. Gustafson, P. Johnson, M.R. Kuehn, S. Lipkowitz, and M.E. Perry for invaluable discussions and comments on this manuscript, and H. Biebuyck for critical advice on high-throughput screening.

This study is dedicated to the memory of our beloved friend and colleague Dr. Christopher J. Michejda.

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