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Protein cross-linking as a novel mechanism of action of a ubiquitin-activating enzyme inhibitor with anti-tumor activity

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ABSTRACT

Ubiquitin-activating enzyme 1 (UBE1) is a critical regulator of the ubiquitination cycle and its targeted inhibition may be an appropriate therapeutic strategy as tumor cells are reported to have increased dependence on protein ubiquitination. PYR-41 is a small molecule with previously described UBE1 inhibitory activity. PYR-41 blocks ubiquitination reactions but paradoxically leads to the accumulation of high MW ubiquitinated proteins. Detailed evaluation of PYR-41 activity demonstrated that PYR-41 inhibited UBE1 activity but also had equal or greater inhibitory activity against several deubiquitinases (DUBs) in intact cells and purified USP5 *in vitro*. Both UBE1 and DUB inhibition were mediated through PYR-41-induced covalent protein cross-linking which paralleled the inhibition of the target proteins enzymatic activity. PYR-41 also mediated cross-linking of specific protein kinases (Bcr-Abl, Jak2) to inhibit their signaling activity. Chemical reactivity modeling provided some insight into the cross-linking potential and partial target selectivity of PYR-41. Overall, our results suggest a broader range of targets and a novel mechanism of action for this UBE1 inhibitor. In addition, since PYR-41-related compounds have demonstrated anti-tumor activity in animal studies, partially selective protein cross-linking may represent an alternate approach to affect signal transduction modules and ubiquitin cycle-regulatory proteins for cancer therapy.

1. Introduction

Protein ubiquitination involves a complex enzymatic cascade through which ubiquitin molecules are covalently linked to target proteins. Ubiquitin-activating enzyme (UBE1/E1) catalyzes the prime reaction in this cascade by 'activating' free monomeric ubiquitin through an ATP-dependent adenylation reaction. The adenylated-ubiquitin is then transferred from E1 to a ubiquitin-conjugating enzyme (UBC/E2), which subsequently transfers ubiquitin to the target protein through ubiquitin-ligases (E3). E3 ligases confer substrate specificity to the ubiquitination pathway by recognizing target substrates and mediating transfer of ubiquitin from an E2 conjugating enzyme to a substrate. There are estimated to be >1000 E3 ligases that can be broadly classified into two families: Homology to E6AP C-Terminus (HECT) domain E3 ligases and Really Interesting New Gene (RING) domain E3 ligases. The HECT and RING family E3 ligases possess distinct mechanisms for substrate ubiquitination. In the case of HECT E3 ligases, activated ubiquitin is transferred from an E2 to an E3 and finally to the lysine residue of a substrate protein. However, RING E3 ligases act by bringing the E2–ubiquitin complex and substrate into close proximity, facilitating the transfer of activated ubiquitin from the E2 directly to the substrate [1]. Ubiquitination has emerged as a key post-translational event which regulates protein degradation [2], signal transduction pathways [3], DNA damage repair [4] and apoptosis [5]. Deubiquitinating enzymes (DUBs) remove ubiquitin molecules from target proteins and thereby primarily serve to counterbalance the ubiquitin–protein conjugation pathway [6]. Human genome codes for 100 putative DUBs which have been divided into four major families: UBP or USP (ubiquitin-specific processing proteases), UCH (ubiquitin carboxy terminal hydrolases), JAMM (Jad1/Pad/MPN-domain-containing metallo-enzymes) and OTU (Otu-domain ubiquitin–aldehyde-binding proteins) [7]. A protein's activity, stability, localization as well as other cellular functions are, therefore, tightly regulated via a fine balance between ubiquitination and deubiquitination.

Cancer cells exhibit alterations in protein ubiquitination with a corresponding change in the activity or half-life of specific

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oncoproteins, which can contribute to tumorigenesis [8–10]. Recently, leukemic cells were shown to exhibit increased levels of ubiquitinated proteins as well as relatively high proteasomal activity when compared to normal peripheral blood mononuclear cells (PBMCs) [11]. Although components of the ubiquitination/deubiquitination pathway are present in both malignant and untransformed cells, the clinical success of bortezomib (proteasome inhibitor) in the treatment of myeloma and lymphoma [12] validates the protein-ubiquitination pathway as an attractive therapeutic strategy. Small molecules which affect oncoprotein ubiquitination and induce tumor cell apoptosis through alterations in this pathway are currently in different stages of development. Inhibitors of E3 ligases such as TRAF6 and MDM2 have been shown to induce tumor cell apoptosis by interfering with NF-κB and p53 signaling, respectively [13–15]. Small molecule inhibitor of NEDD8 activating enzyme (NAE), MLN4924, is being currently evaluated in several phase I clinical trials. MLN4924 was shown to selectively inhibit NAE activity and exhibit potent against a variety of human tumor-derived cell lines [16,17]. DUB inhibitors provide an alternate approach to interfere with the ubiquitin regulatory cycle [18]. Cyclopentenone prostaglandins were among the first described cell permeable inhibitors of cellular DUBs [19]. Based on the presence of a common structural determinant, DUB inhibitors such as dibenzylideneacetone (DBA), curcumin and shikoccin, were described [20]. We recently described the DUB inhibitory activity of WP1130, a novel tyrphostin analogue, which alters ubiquitination of specific proteins, leading to tumor cell apoptosis [21].

UBE1 catalyzes the first and critical step in the protein ubiquitination pathway and cell permeable inhibitors of UBE1 have been developed as potential anti-tumor agents [11,22]. 4-[4-(5-Nitro-furan-2-ylmethylene)-3,5-dioxo-pyrazolidin-1-yl]-benzoicacid ethyl ester (PYR-41) is the first reported, cell permeable, potent inhibitor of UBE1 [22]. PYR-41 was also reported to selectively induce apoptosis in transformed cells. Formation of a covalent-linkage between the active site cysteine of UBE1 and PYR-41 was hypothesized to be its mechanism of action. PYR-41 has been widely used as a UBE1 inhibitor to inhibit ubiquitination reactions, as reported in several publications.

Based on its previously defined activity against UBE1, we used PYR-41 to inhibit WP1130-induced ubiquitination in our mechanism of action studies. We consistently noted accumulation of very high MW ubiquitinated proteins (>250 kDa) in cells treated with PYR-41, even after potent UBE1 inhibition. Yang et al. [22] also described this phenomenon but reported no net change in total cellular ubiquitinated protein in PYR-41 treated cells. We investigated the mechanism of action of PYR-41 and show that DUB activity from PYR-41 treated cells was significantly reduced at concentrations lower than those needed for UBE1 inhibition. PYR-41 showed significant inhibition of USP5 activity even at low μM concentrations in an *in vitro* enzyme assay. We further show that PYR-41 led to a decline in cellular protein levels of DUBs such as USP5 and USP9x, which was independent of their proteasomal degradation. Further analysis demonstrated that PYR-41 induces protein cross-linking, leading to formation of high MW target protein adducts. These results demonstrate that PYR-41 inhibits UBE1 activity but also inhibits other ubiquitin regulatory enzymes and signal transducing proteins through chemical cross-linking.

2. Materials and methods

2.1. Cell culture, chemical reagents and enzymes

Z138 (mantle cell lymphoma), K562 (chronic myelogenous leukemia) and H929 (multiple myeloma) cells were grown in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Human embryonic kidney 293T (HEK293T) cells were cultivated in Dulbecco modified essential medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) containing 10% FBS. PYR-41 was purchased from Biogenova (Rockville, MD). Other reagents used in this study were obtained from the following sources: MG-132, NEM (N-ethylmaleimide), ubiquitin–agarose, Ub-AMC, hemagglutinin-tagged ubiquitin vinyl methyl sulfone (HA-UbVS), Suc-LLVY-AMC, UBE1, USP5 (BostonBiochem, Cambridge, MA); DSP (Thermo Fisher Scientific, Rockford, IL). WP1130 was synthesized and provided by our co-author (Dr. H. Showalter, University of Michigan).

2.2. Western blotting

Whole cell lysates were prepared by boiling and sonicating the cell pellet in 1× Laemmli reducing sample buffer. Samples were resolved by SDS-PAGE and immunoblotted. Antibodies used in this study were purchased from the following sources: anti-actin (Sigma–Aldrich, St. Louis, MO); anti-ubiquitin clone P4D1, anti-UBE1, anti-Stat3, anti-Stat5, anti-Abl, anti-CrkL, goat anti-rabbit/mouse/rat IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA); anti-USP9x, anti-USP5, anti-Jak2 (Bethyl Laboratories, Montgomery, TX); anti-RUNX1 (Novus Biologicals, Littleton, CO); anti-HA [clone 3F10] (Roche Applied Science Indianapolis, IN); anti-pStat3, anti-pCrkL (Cell Signaling, Danvers, MA).

2.3. Ubiquitin–agarose pull down

Cells were left untreated or treated with WP1130 (5 μM) or PYR-41 (50 μM) alone or with the combination of both agents for 2 h. Binding assays were performed by incubating lysates prepared in UBE1 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 1% Triton X-100) with 20 μl of ubiquitin–agarose for 2 h at 4 °C. The beads were washed extensively with UBE1 buffer and the bound proteins resolved by SDS-PAGE and immunoblotted with anti-UBE1 antibody.

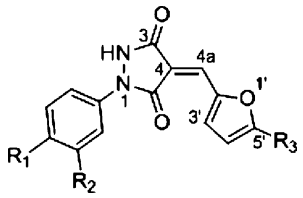
2.4. Proteasome activity assay

To assay for *in vivo* proteasome inhibition, Z138 cells were treated with PYR-41 (50 μM) or MG132 (5 μM) for 2 h. The cells were lysed in ice-cold lysis buffer (50 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100) and their lysates clarified by centrifugation at 20,000 RCF for 10 min. Equal amounts of protein from each sample were then incubated at 37 °C with 100 μM fluorogenic substrate (Suc-LLVY-AMC). Fluorescence intensity was measured at excitation 360 nm and emission 460 nm. Assays were performed in triplicate, and statistical significance was determined with a paired Student's *t* test.

2.5. Ub-AMC protease assay

Cells were lysed in ice cold DUB buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM sucrose, 0.5 mM DTT, 5 mM $MgCl_2$ and 1 mM phenylmethylsulfonylfluoride by mild sonication. Briefly, 5–10 μg of clarified lysate from untreated and PYR-41 treated cells were incubated with 500 nM Ub-AMC in a 100 μl reaction volume at 37 °C and the release of AMC fluorescence per minute was recorded at em/em 380/460 nm. Purified USP5 (50 nM) was incubated in DUB buffer containing PYR-41, vehicle alone (DMSO) or 1 mM NEM (positive control for DUB inhibition) in a 100 μl reaction volume for 30–60 min at 37 °C. The reaction was initiated by the addition of 500 nM Ub-AMC and the release of AMC-fluorescence was recorded at ex/em 380/480.

Table 1
Calculated LUMO and HOMO values for literature pyranones.



Compound	R_1	R_2	R_3	LUMO	HOMO	Gap
PYR-41	$-CO_2Et$	H	NO_2	0.0066	−0.3222	0.3288
PYR-19	H	H	H	0.0376	−0.3002	0.3378
PYR-823	H	Br	NO_2	0.0034	−0.3238	0.3272
PYR-495	Br	H	H	0.0316	−0.3036	0.3352
PYZD-4409	F	Cl	NO_2	0.0122	−0.3230	0.3352
PYZD-4409_mut	Me	H	H	0.0386	−0.2932	0.3318

HOMO and LUMO energy levels (Hartrees) of the literature pyranones computed, using the GAMESS [24] interface through ChemBio3D Ultra 12.0 [25] with the Restricted Hartree–Fock [26] method and the 6-31+G(d,p) basis set [27].

2.6. Deubiquitinase labeling assays

To assay for changes in activity of cellular deubiquitinase enzymes, Z138 cells were lysed in DUB buffer for 10 min at 4 °C. The lysates were centrifuged at 20,000 RFC for 10 min and the supernatant was used for DUB labeling as previously described [23,24]. Equal amounts of lysate were incubated with 200 nM of HA-UbVS for 1 h at room temperature, followed by boiling in reducing sample buffer and resolving by SDS-PAGE. HA immunoblotting was used to detect DUB labeling.

2.7. Protein cross-linking assay

Purified USP5 (200 ng) was incubated in DUB buffer (without DTT) containing DMSO, PYR-41 or NEM. After 30 min of incubation at 37 °C, 200 nM HA-UbVS was added to all samples (except the negative control) and the reaction was further incubated for 1 h at 37 °C. The activity of USP5 was measured by HA immunoblotting. The membranes were then probed with anti-USP5. The effect of DTT on the cross-linking activity of PYR-41 was examined by incubating UBE1 (250 nM) or USP5 (200 ng) in DUB buffer containing DMSO or PYR-41 in the presence or absence of 5 mM DTT. Protein cross-linking was detected by immunoblotting with UBE1 or USP5 antibodies.

To compare the cross-linking activity of PYR-41 to the chemical cross-linker DSP, USP5 (200 ng) was incubated in DUB buffer containing DMSO, PYR-41 or DSP for 30 min at 37 °C. The samples were resolved using non-reducing or reducing SDS-PAGE and the cross-linking effects of each compound were examined by USP5 immunoblotting.

Jak2 was immunoprecipitated from HEK293 cells and the beads were washed extensively in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA). The beads were incubated in 50 μl of lysis buffer containing DMSO, 50 μM PYR-41 or 50 μg/ml DSP for 30 min at 37 °C. The beads were then boiled in reducing Laemmli buffer (non-reducing for DSP) and the samples were resolved by SDS-PAGE. Jak2 cross-linking was assessed by immunoblotting with anti-Jak2 antibody.

2.8. Ab initio calculations

Low energy conformations were first generated for each of the structures in MOE [25] using a systematic conformational search with energy minimization and MMFF94 parameters [26,27]. The lowest energy conformations for this series had the furan oxygen pointed away from the central ring as illustrated in Table 1. The lowest energy conformation for each structure was used as a starting point for the *ab initio* calculations. The structures were further minimized and the HOMO and LUMO energy levels computed, using the GAMESS [28] interface through ChemBio3D Ultra 12.0 [29] with the Restricted Hartree–Fock [30] method and the 6-31+G(d,p) basis set [31]. The computed HOMO, LUMO, and band-gap energy levels are shown for PYR-41 and its congeners in Table 1.

3. Results

3.1. Accumulation of high MW ubiquitinated proteins in cells treated with PYR-41

We recently described the DUB inhibitory activity of WP1130 (analogue of tyrphostin AG-490), which induces accumulation of polyubiquitinated proteins and apoptosis in tumor cells [21]. Since UBE1 mediates the critical first reaction in the protein–ubiquitin conjugation pathway [2], we hypothesized that pretreatment with the UBE1 inhibitor PYR-41 would prevent WP1130-induced accumulation of ubiquitinated proteins. Z138 cells were pretreated with 50 μM PYR-41 for 30 min, followed by further 1 h incubation in the presence or absence of 5 μM WP1130 before assessing cellular ubiquitination by immunoblotting. As shown in Fig. 1A, WP1130 treatment induced accumulation of ubiquitinated proteins (compare lanes 1 and 2) which was suppressed by pretreatment with PYR-41 (compare lanes 2 and 4). However, very high MW ubiquitinated proteins (>250 kDa and abundant in the stacking gel) were observed in PYR-41 treated cells (lanes 3 and 4). To verify that PYR-41 effectively inhibited UBE1 activity in these cells, an additional cell lysate prepared in UBE1 buffer was probed for the amount of UBE1 bound to a Ub–agarose matrix. As shown in Fig. 1A (lower panel), pretreatment with PYR-41 inhibited UBE1 activity as determined by a loss of UBE1 ubiquitin–agarose binding activity. The observation that PYR-41 can lead to an accumulation of high molecular weight ubiquitinated proteins while inhibiting UBE1 activity was unexpected but previously described (Fig. 3B in [22]). Accumulation of ubiquitinated proteins in response to PYR-41 treatment was detected in other cell types as well, including K562, H929 and HEK293 (Fig. 1B). Given this result, we assessed the effect of PYR-41 on 20S proteasome activity in Z138 cells but found no significant decline in 20S proteasome core-subunit activity in cells treated with PYR-41 (Fig. 1C).

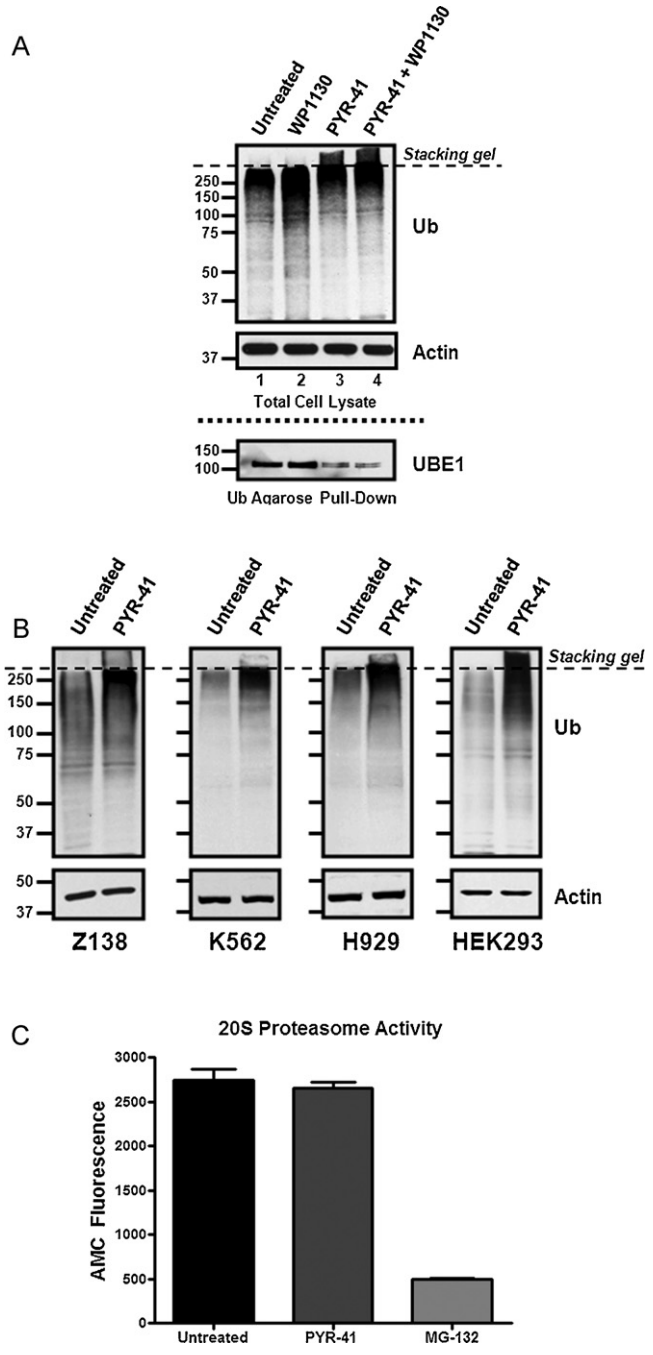


Fig. 1. PYR-41 treatment promotes accumulation of ubiquitinated proteins. (A) Z138 cells were left untreated or treated with WP1130 (5 μM), PYR-41 (50 μM) or a combination of both agents for 2 h. The cells were split in two fractions; the first fraction was lysed in Laemmli buffer and probed to detect changes in protein ubiquitination (top). Actin was immunoblotted as a protein loading control. The second fraction was lysed in UBE1 buffer and processed for ubiquitin–agarose pull down assays as described in Section 2 (bottom). (B) Z138 (lymphoma), K562 (leukemia), H929 (myeloma) and HEK293 (embryonic kidney) cells were left untreated or treated with 50 μM PYR-41 for 2 h before cell lysates were subjected to Ub immunoblotting. Actin was probed as a protein loading control. (C) Lysates from untreated, PYR-41 (50 μM) or MG-132 (5 μM) treated Z138 cells were assayed for 20S core subunit proteasome activity as described in Section 2. The results represent the average ± SD of triplicate assays.

3.2. PYR-41 inhibits deubiquitinase activity in vivo and in vitro

Since increased cellular ubiquitination can occur through suppression of deubiquitinating enzymes (DUB) [19,32] we examined the effect of PYR-41 treatment on cellular DUB activity. Ubiquitin-AMC is a DUB substrate that releases fluorescence when AMC fluorophore is cleaved from the ubiquitin molecule by an active DUB [33]. Lysates derived from untreated or PYR-41 (50 μM, for 1–4 h) treated Z138 cells were incubated with ubiquitin-AMC and fluorescence was monitored as described in Section 2. We observed a time-dependent decline in DUB activity/AMC-fluorescence in lysates obtained from PYR-41 treated Z138 cells with >95% inhibition observed after 4 h at 50 μM PYR-41 (Fig. 2A). PYR-41 caused a concentration-dependent (10–50 μM) decline in DUB activity in Z138 cells after 4 h (Fig. 2B). To further validate DUB inhibitory activity by PYR-41, we incubated purified USP5 with various doses of PYR-41 (10–50 μM) for 30 min prior to the addition of ubiquitin-AMC and monitored changes in USP5 activity. As shown in Fig. 2C, PYR-41 potently inhibited USP5 DUB activity, even at the lowest concentration examined (10 μM).

3.3. Effect of PYR-41 on DUB protein levels

To further assess the profile of cellular DUBs that may be affected by PYR-41, we used the strategy first described by Borodovsky et al. [23,24]. HA-UbVS is a potent, active-site directed, irreversible inhibitor of DUBs. Active DUBs bind HA-UbVS covalently and can be detected by HA immunoblotting [23,34,35]. We treated Z138 cells with various concentrations of PYR-41 (10–50 μM) or 2 mM NEM (known DUB inhibitor) for 1 h and the cells were lysed and processed for DUB labeling as described in Section 2. Immunoblotting with anti-HA antibody revealed PYR-41 potently inhibited the activity of various DUBs, determined to represent USP9x, USP5, USP14, UCH37 and UCH-L3 (Fig. 2D) (based on mass-spectrometric analysis of tagged DUBs) [18,34]. These results are aligned with the results obtained in DUB activity assays (Fig. 2A–C). We also observed an apparent dose-dependent decline in the USP9x and USP5 protein level by immunoblotting and used proteasome inhibitor (MG-132) to determine whether this loss was due to their proteasomal degradation. As shown in Fig. 2E, treatment of Z138 cells with PYR-41 in the presence of MG-132 did not block the reduction of USP9x and USP5 protein levels. However, very high MW species of USP9x and USP5 (denoted by *) were detected in PYR-41 treated cells (lanes 3 and 4). These results suggest that PYR-41 induces a conformational change in DUB proteins, which leads to their loss of activity and change in electrophoretic mobility.

3.4. PYR-41 covalently cross-links DUBs to suppress their activity

To determine whether PYR-41 induces formation of high MW adducts of cellular proteins, Z138 cells were treated with PYR-41 (10–50 μM) for 1 h and whole cell lysates were prepared by boiling and sonicating in reducing Laemmli buffer. PYR-41 treatment led to the formation of high MW species/adducts (denoted by *) of USP9x and UBE1 in a concentration-dependent fashion. However, we also observed high MW species of RUNX1 (Fig. 3A), which is not involved in regulating the protein ubiquitination cascade.

PYR-41 was previously reported to induce SUMOylation of proteins which was associated with the inhibition of UBE1 activity [22]. However, PYR-41 directly inhibits purified USP5 activity, eliminating the possible involvement of a post-translational inhibitory mechanism. This observation suggested the possibility for direct chemical reactivity of PYR-41 with target proteins to affect their activity. To determine if PYR-41 promotes cross-linking of proteins, an *in vitro* cross-linking assay was performed using USP5 as a substrate. As shown in Fig. 3B, PYR-41 treatment inhibited USP5 labeling activity in a dose-dependent fashion (HA-immunoblotting), which directly correlated with the formation of high MW USP5 species. Dithiobis succinimidyl propionate (DSP; 50 μg/μl) is a chemical agent that promotes protein cross-linking.

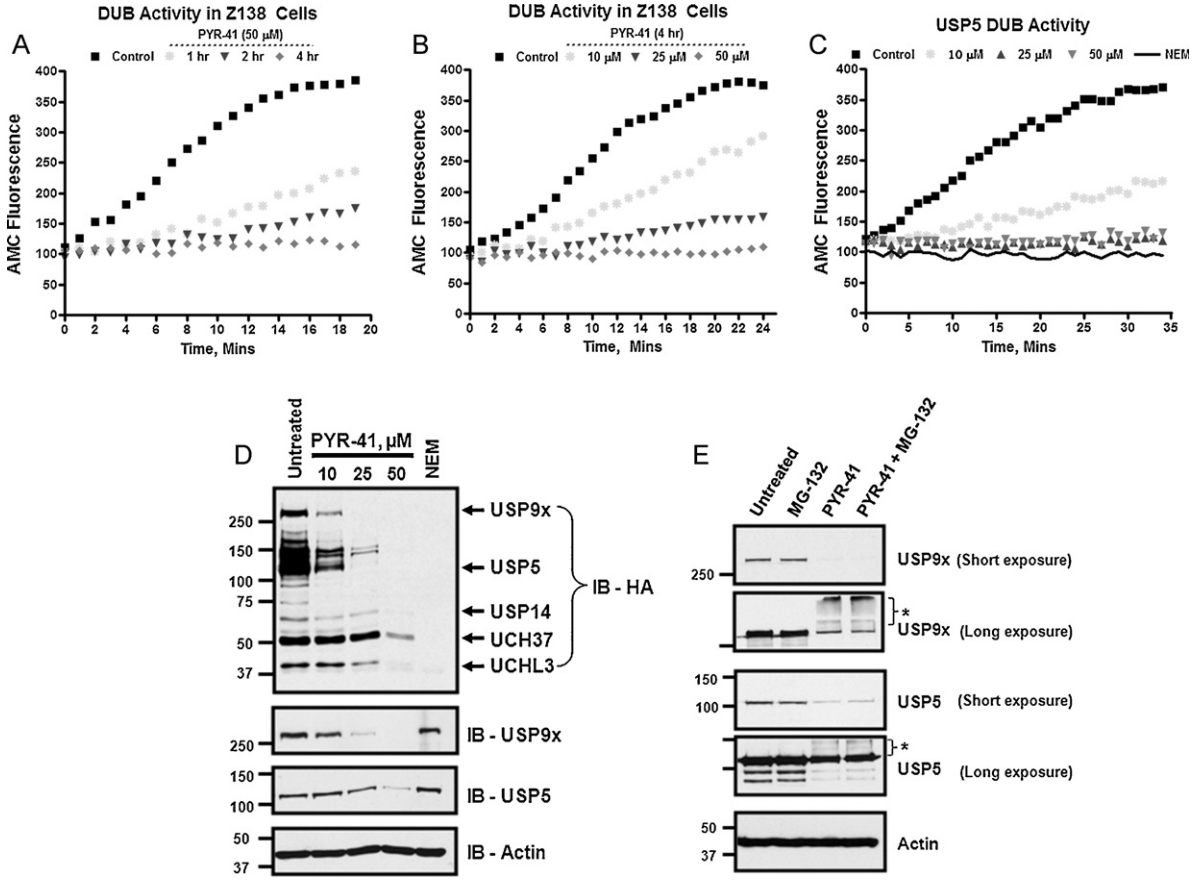


Fig. 2. Inhibition of DUB activity by PYR-41. (A) Z138 cells were left untreated or treated with 50 μM PYR-41 for the indicated interval and lysed in DUB buffer. 10 μg of lysate from these cells was probed for DUB activity using ubiquitin-AMC as described in Section 2. The results represent the result from a single experiment. Similar results were obtained in 2 additional independent experiments. (B) Z138 cells were left untreated or treated with the indicated concentration of PYR-41 for 2 h and lysed in DUB buffer. DUB activity assays were performed as described in Section 2. The results represent the result from a single experiment. Similar results were obtained in 2 additional independent experiments. (C) Purified USP5 (Boston Biochem; 50 nM) was incubated with DMSO or the indicated concentration of PYR-41 in DUB buffer for 30 min at 37 °C in a 100 μl reaction volume. USP5 activity was assessed by ubiquitin-AMC assay as described in Section 2. NEM is a positive control for USP5 inhibition. The results represent the result from a single experiment. Similar results were obtained in 2 additional independent experiments. (D) Z138 cells were treated with the indicated concentration of PYR-41 for 2 h, followed by their lysis in DUB buffer. 20 μg of lysate was probed for changes in cellular DUB activity by the addition of 200 nM HA-UbVS and incubation for 1 h at 37 °C. Lysates were resolved by SDS-PAGE and immunoblotted with anti-HA. Membranes were stripped and re-probed for USP9x, USP5 and actin (loading control). (E) Z138 cells were pre-treated with 5 μM MG-132 for 1 h, followed by further incubation in the presence or absence of 50 μM PYR-41 for 2 h. Lysates were probed for USP9x, USP5 and actin (loading control). USP9x and USP5 adducts were detected on prolonged exposure (* denotes high MW adducts).

Purified USP5 was incubated in the presence of DSP (50 μg/μl) or PYR-41 (50 μM) and resolved using non-reducing and reducing SDS-PAGE. The disulfide bond in the spacer arm of DSP is sensitive to reducing agents, allowing us to interrogate possible similarities between PYR-41 and DSP cross-linked activity. As shown in Fig. 3C, both DSP and PYR-41 led to the formation of high MW USP5 adducts. However, unlike DSP-induced cross-linked USP5 adducts, PYR-41 induced cross-linked USP5 species were resistant to reducing agent (β-mercaptoethanol, compare lanes 5 and 6). These results suggested that PYR-41 induces formation of covalent, heat and reducing agent stable multimers of cellular proteins.

3.5. PYR-41 activity is inactivated by DTT

PYR-41 mediated inhibition of UBE1 was previously shown to be sensitive to thiol containing compounds, such as GSH. This suggests the possibility that PYR-41 modifies its target protein/s by interaction with cysteine residues. We therefore assessed whether the accumulation of high MW ubiquitinated proteins in PYR-41 treated cells could be blocked in the presence of DTT. As shown in Fig. 4A, co-treatment of Z138 cells with DTT and PYR-41 completely abolished the accumulation of ubiquitinated proteins (compare lanes 4–6). High MW adducts of USP9x, USP5 and RUNX1, induced by PYR-41 treatment, were also inhibited in the presence of DTT (Fig. 4A). As expected, no high MW adducts of USP5 were detected when purified USP5 was incubated with PYR-41 in the presence of DTT (Fig. 4B, compare lanes 2 and 4). Similar results were also obtained in an *in vitro* reaction of UBE1 with PYR-41 and DTT.

3.6. Suppression of oncogenic signaling by PYR-41

Based on our observations of the off-target effects of PYR-41, we hypothesized that incubation with PYR-41 might also inhibit cellular signaling by cross-linking protein kinases involved in oncogenesis. We investigated the effect of PYR-41 on IL-6 driven Jak2-Stat3 signaling. As shown in Fig. 5A, treatment of HEK293 cells with PYR-41 completely abolished IL-6 dependent activation of Stat3. This was primarily associated with formation of high MW adducts of Jak2 detected in western blots. PYR-41 also induced formation of high MW adducts of Jak2 in an *in vitro* reaction (Fig. 5B), further illustrating the scope of cross-linkable targets for PYR-41.

CML cells are primarily engaged in oncogenic signaling through the tyrosine kinase activity of the Bcr-Abl fusion protein [36]. To investigate the effects of PYR-41 on Bcr-Abl signaling, we

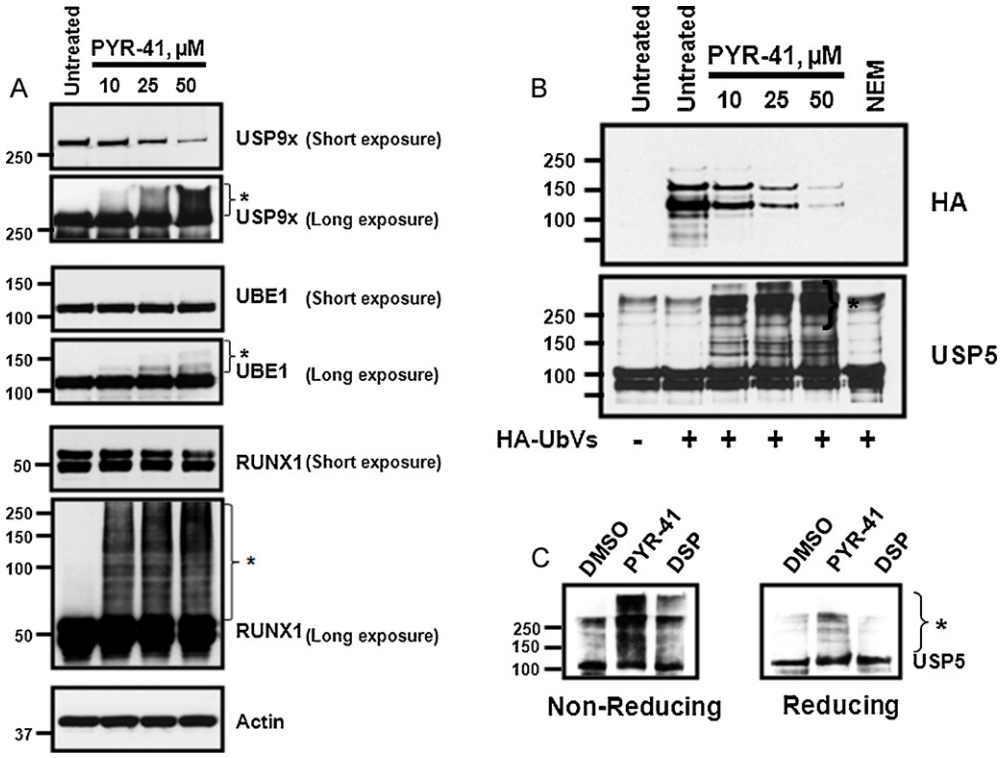


Fig. 3. PYR-41 covalently cross-links cellular proteins. (A) Z138 cells were left untreated or treated with the indicated concentration of PYR-41 for 1 h. Lysates were prepared in reducing Laemmli buffer and probed for USP9x, UBE1, RUNX1 and actin (loading control). Long-exposure to autoradiography film shows concentration-dependent accumulation of high MW adducts of USP9x, UBE1 and RUNX1 (* denotes high MW adducts). (B) *In vitro* USP5 cross-linking analysis was performed as described in Section 2 in the presence of increasing concentrations of PYR-41. Changes in USP5 activity were assessed by HA-UbVS labeling and HA immunoblotting. High MW adducts of USP5 were detected by USP5 immunoblotting. (C) Purified USP5 was incubated in DUB buffer containing DMSO, PYR-41 (50 μM) or DSP (50 μg/ml) for 30 min at 37 °C. The samples were resolved using non-reducing (*left*) and reducing (*right*) SDS-PAGE and blotted with anti-USP5 antibody to detect formation of high MW USP5 species.

incubated K562 cells with 5 μM imatinib (Bcr-Abl kinase inhibitor) or 50 μM PYR-41 for 2 h. As shown in Fig. 5C, very high MW adducts of Bcr-Abl protein were detected in lysates from PYR-41 treated cells, suggesting PYR-41 induces cross-linking of Bcr-Abl as well. In contrast, no change in Bcr-Abl protein levels were detected in imatinib treated cells. Though a major impact of PYR-41 on Bcr-Abl protein level was detected, we did not see a significant decline in phosphorylation of its downstream substrates (Stat5, CrkL, see Section 4).

4. Discussion

In this manuscript, we describe the mechanism of action and multi-target activity of PYR-41, initially described as a cell-permeant UBE1 inhibitor with impact on many signaling cascades activated in tumor cells. Based on a chemical library of PYR-41 related compounds containing the pyrazolidine pharmacophore, a novel derivative (3,5-dioxopyrazolidine compound, PYZD-4409) was recently identified as another UBE1 inhibitor and shown to exhibit anti-leukemic activity [11]. As a proof of principle, shRNA mediated knock-down of UBE1 was shown to preferentially induce apoptosis of leukemic cells versus normal peripheral blood mononuclear cells [11]. These observations support the potential for UBE1 inhibitors to alter ubiquitination patterns in tumor cells to induce apoptosis. However, the results obtained in the present study suggest that PYR-41 is an unspecific inhibitor of many cellular proteins.

PYR-41 treated cells showed a potent, time- and concentration-dependent inhibition of cellular DUB activity (Fig. 2A and B). In mantle cell lymphoma cells, PYR-41 showed significant inhibition of various DUBs, as assessed in HA-UbVS DUB labeling experiments (Fig. 2D) and also noted an apparent decline in DUB protein levels (USP9x/USP5). Since PYR-41 was previously shown to stabilize cellular proteins such as p53 and cyclin D1 by blocking their ubiquitination-dependent proteasomal degradation, the loss of DUB proteins in response to PYR-41 treatment was unexpected. To investigate whether the effects of PYR-41 were limited to proteins of the ubiquitination pathway, we probed for changes in the electrophoretic mobility of unrelated proteins such as RUNX1, Jak2 and Bcr-Abl and detected the presence of high MW adducts of each protein in PYR-41 treated cells (Figs. 3A and 5A–C). We conclude that the apparent decline in detection of these proteins at their nominal MW was due to PYR-41-induced formation of high MW adducts. This activity partially explains the PYR-41 mechanism of action.

Recently, 17-hydroxy-jolkinolide B (HJB) was shown to block Jak2 signaling by inducing covalent cross-linking of Jak2 and Jak family members [37] which are no longer able to respond to cytokine stimulation. Although PYR-41 pre-treatment could effectively block cytokine signaling through Jak2, it did not significantly affect signaling downstream of the constitutively active Bcr-Abl kinase (Stat5, CrkL phosphorylation) despite PYR-41-mediated Bcr-Abl cross-linking. This may be due to the simultaneous inactivation or cross-linking of phosphatases which control dephosphorylation of these substrates. It should also be noted that while PYR-41 was shown to *enhance* signaling pathways which are negatively regulated through ubiquitination [22], shorter (5–15 min) PYR-41 pre-incubation intervals were used prior to brief cytokine exposure and may have distinct effects when compared to the longer incubation intervals used in this study. We conclude that although PYR-41 incubation leads to inhibition of cellular UBE1 activity, prolonged or sustained PYR-41 incubation will ultimately result in unspecific protein cross-linking.

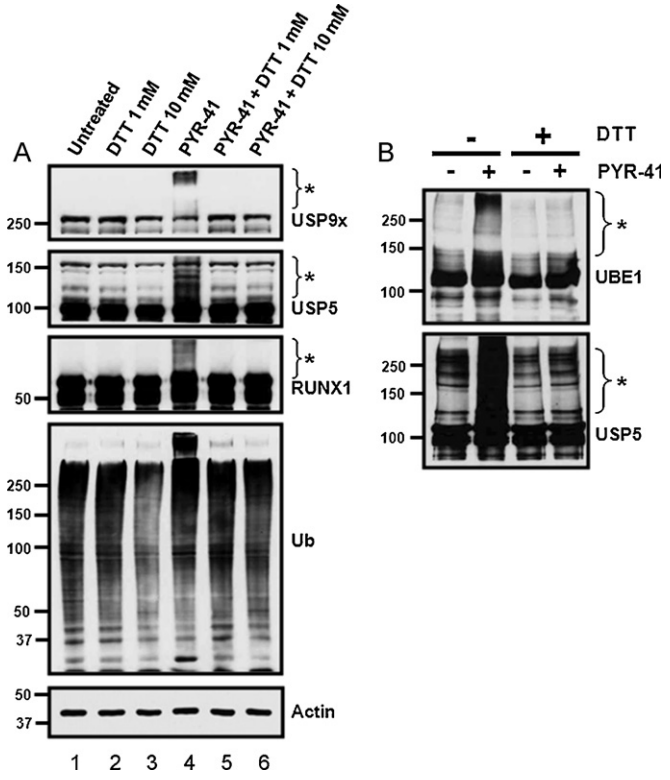


Fig. 4. Effects of PYR-41 are DTT-sensitive. (A) Z138 cells were treated with 1 mM or 10 mM DTT alone or in the presence of 50 μM PYR-41 for 2 h. Z138 cells treated with PYR-41 (50 μM) alone for 2 h acted as positive control for the formation of high MW adducts of cellular proteins, detected by immunoblotting USP9x, USP5, RUNX1, and Ub. Actin was probed as a protein loading control (* denotes high MW adducts). (B) Purified USP5 (50 nM) or UBE1 (200 nM) were incubated in DUB buffer/UBE1 buffer respectively, in the presence or absence of 50 μM PYR-41 and 5 mM DTT as indicated. The samples were incubated at 37 °C for 1 h, and the formation of high MW adducts of USP5/UBE1 were probed by immunoblotting (* denotes high MW adducts).

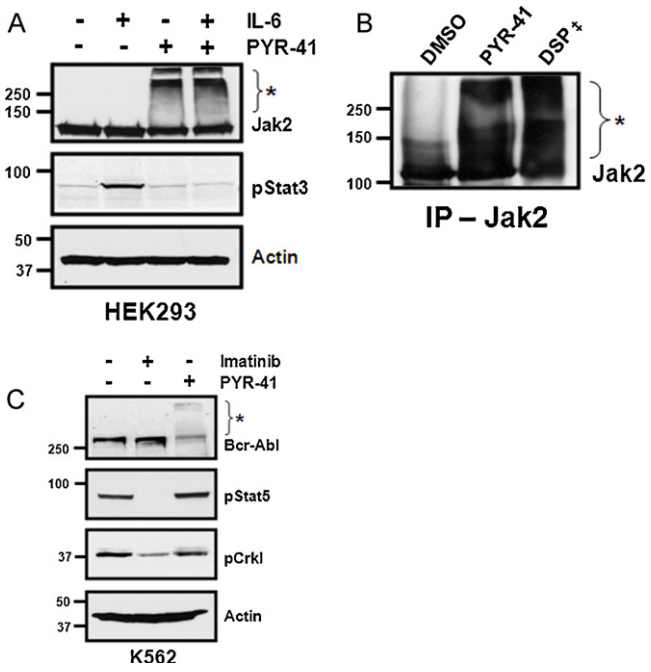


Fig. 5. PYR-41 suppresses cellular signaling pathways through kinase cross-linking. (A) HEK293 cells were left untreated or stimulated with IL-6 (20 ng/mL) for 15 min to activate the Jak2-Stat3 pathway. HEK293 were also incubated with PYR-41 (50 μM) alone for 2 h or PYR-41 (2 h) + IL-6 (15 min). The lysates were resolved by SDS-PAGE and the activation of Jak2 signaling assessed by immunoblotting with pStat3 (Y705). Membranes were also probed for Jak2 and actin (* denotes high MW adducts). (B) Jak2 was immunoprecipitated from HEK293 cells and the agarose beads were washed extensively using lysis buffer. The beads were then incubated in the presence of DMSO, PYR-41 (50 μM) or DSP (50 μg/ml) for 30 min at 37 °C. The samples were resolved by SDS-PAGE and blotted with anti-Jak2 antibody. ‡DSP cross-linked sample was boiled in non-reducing sample buffer (* denotes high MW adducts). (C) K562 cells were left untreated or treated with imatinib (5 μM) or PYR-41 (50 μM) for 2 h. Lysates were probed for Bcr-Abl, pStat5, pCrkL and actin (* denotes high MW adducts).

PYR-41 contains an α,β-unsaturated enone moiety with a sterically accessible β-carbon. α,β-Unsaturated enones react rapidly with sulfhydryl containing compounds such as GSH and DTT through a Michael addition reaction [38]. We observed complete inhibition of PYR-41 induced protein cross-linking activity (*in vivo* and *in vitro*) in the presence of DTT. The inability of PYR-41 to inhibit UBE1 activity or to induce protein–protein cross-linking in the presence of DTT (or GSH) suggests that the presence of the α,β-unsaturated enone in PYR-41 may account for its protein cross-linking abilities. Previous investigators examining the mechanism of UBE1 enzyme inhibition by PYR-41 have suggested that its structure renders it a target for nucleophilic attack, potentially by an active site cysteine [22]. Attack was invoked as occurring either by reaction with the N-1 aryl bond, Michael addition to the C-4a exocyclic double bond, or interaction with the C-5 nitro function (Fig. 6A; arrows a–c, respectively). Inhibition of UBE1 by PYR-41 was shown to be completely abolished in the presence of excess glutathione (GSH). Another key observation was complete selectivity due to the "nitro effect" of the furan ring, but no explanation was offered. We examined the consequence of DTT/PYR-41 incubation on generation of alternate species by time-of-flight mass spectroscopy. We saw peaks we can ascribe to the presence of PYR-41, a single DTT adduct due to a Michael addition, and an adduct due to a double addition of DTT (Michael addition plus displacement of the nitro group). These preliminary results need to be confirmed by additional analysis but are aligned with potential sites of reactivity predicted from *ab initio* calculations.

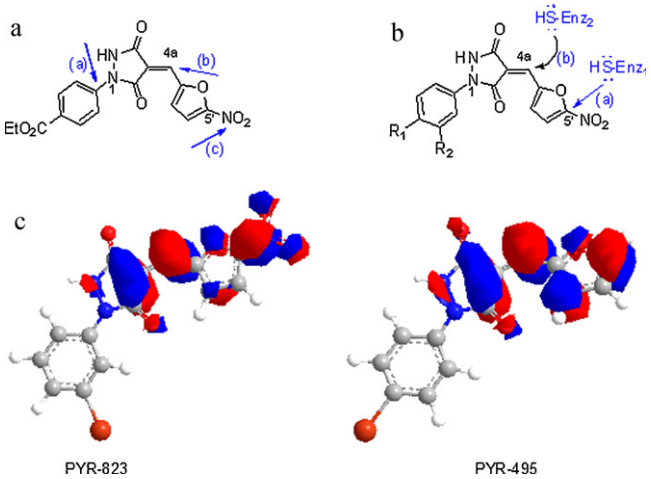


Fig. 6. Mechanistic models of E1 and DUB enzyme inhibition by 5-nitropyranones. (A) Proposed model of PYR-41-mediated UBE1 inhibition/enzyme trapping according to Yang et al. [22]. Possible sites are shown by arrows a–c. (B) Proposed model of intermolecular cross-linking in which a cysteine residue first displaces the nitro group at the C-5′ position (arrow a) followed by Michael addition of a second cysteine to the C-4a position (arrow b). (C) Graphical representations of the LUMO orbitals for PYR-823 and PYR-495 computed and displayed by ChemBio3D Ultra 12.0 [29] with extended Hückel calculations. Red versus blue coloring indicates the different algebraic signs of the wave functions, not electrostatic charge.

We used *ab initio* calculations to compute the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) energy levels for PYR-41 and its more recently described congeners [11] to better understand these observations and to offer a plausible mechanism for the formation of cross-linked proteins observed in our studies. Low energy conformations were first generated for each of the structures in MOE [25] using a systematic conformational search with energy minimization and MMFF94 parameters [26,27]. HOMO, LUMO, and band-gap energy levels were computed for each compound (Table 1). The lowest energy conformation for this series has the furan oxygen pointed away from the central ring. LUMOs for the structurally similar nitro (PYR-823) and des-nitro (PYR-495) pair are illustrated in Fig. 6C and are representative of the LUMOs for each of the structures in the series. The first available molecular orbital capable of accepting an electron pair by definition is the LUMO. The lower (i.e., more negative) the LUMO's energy, the greater its potential to act as an electron acceptor. The location of the LUMO in Fig. 6C is centered at the exocyclic C-4/C-4a double bond for each compound, suggesting the potential for a Michael addition by an external nucleophile at this position. While the computed LUMO energy levels in Table 1 are lowest for nitro compounds (and thus more favorable to Michael addition), we believe that adducts can form readily for all listed compounds. So the differential LUMO values alone cannot explain the observed experimental selectivity for nitro compounds.

A model rationalizing selective enzyme inhibition in previous studies and cross-linking results for our and prior studies is shown in Fig. 6B. This draws from the observation that even though a large amount of the LUMO is centered on the exocyclic C-4/C-4a double bond for nitro containing structures, there is also considerable LUMO over the C-N bond at C-5′. Thus, we propose that selective enzyme inhibition by nitro pyrazones is due to an *irreversible* trapping of a cysteine residue of UBE1 or DUB enzyme at the C-5′ position α to the nitro group (Fig. 6B, arrow a). Such a reaction is well precedented chemically for furans substituted with an electron-withdrawing function at C-2′ and a nitro group at C-5′ [39]. While Michael addition of a cysteine residue could also occur at the more reactive exocyclic C-4/C-4a double bond for each compound of Table 1, we believe that such a reaction is *reversible* under normal enzymatic or cellular conditions where the concentration of thiol residues relative to inhibitor is low. Further, we believe that our proposed mechanism can rationalize the formation of high molecular weight protein adducts from PYR-41 treatment observed in our and previous studies [22]. In this case, the initial C-5′ thiol adduct (Fig. 6B, arrow a) now alters the LUMO of the exocyclic C-4/C-4a double bond such that a second thiol addition (arrow b) occurs in a fashion leading to intermolecular cross-linked enzymes/proteins. Our mechanism also provides an explanation for our and previous studies [11,22] wherein pretreatment with high levels of thiol (10^4- to 10^5-fold excess relative to enzyme) totally abolished inhibition by nitro pyranones, but not des-nitro congeners. We do not believe that inhibition due to reaction with the N-aryl bond (Fig. 6A, arrow a) plays any role, as this is not chemically precedented. Reaction with the nitro function (arrow c) could play a role in cells, requiring reduction of the nitro function to a reactive species by nitro reductases.

Future structure–activity relationship studies and analysis of the composition of the cross-linked protein complexes will be necessary to conclusively establish the molecular basis of how nitro pyranones inhibit UBE1 and DUB target enzymes. The apparent irreversible nature of UBE1 inhibition, and the quenching of activity by a marked excess of free thiols together provide strong circumstantial evidence that PYR-41 and nitro congeners block an active site cysteine residue(s). Molecular modeling based on a partial structure of UBE1 [40] is consistent with such a mechanism. The unspecific nature of target protein inhibition by PYR-41 may limit its use as an anti-tumor agent. However, it is important to note that new derivatives with structural similarity to PYR-41 have demonstrated anti-leukemic activity in animal studies [11], providing evidence that agents with this unique, but broad mechanism of action, may hold clinical potential.

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