Biochemical Pharmacology 82 (2011) 341–349

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

Vaibhav Kapuria [a,c], Luke F. Peterson [a], H.D. Hollis Showalter [b], Paul D. Kirchhoff [b], Moshe Talpaz [a], Nicholas J. Donato [a,*]

[a] Department of Internal Medicine, Division of Hematology-Oncology, University of Michigan, Ann Arbor, MI, USA
[b] Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI, USA
[c] Graduate School of Biomedical Sciences, University of Texas Health Science Center at Houston, Houston, TX, USA

ARTICLE INFO

Article history:
Received 23 March 2011
Accepted 12 May 2011
Available online 19 May 2011

Keywords:
Ubiquitin-activating enzyme
Deubiquitinase
Inhibitor
Cross-linking

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

* Corresponding author at: Department of Internal Medicine, 1500 E. Medical Center Drive, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI, USA. Tel.: +1 734 615 5542; fax: +1 734 647 9654.
E-mail address: ndonato@med.umich.edu (N.J. Donato).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.05.012

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.