The 19S Deubiquitinase Inhibitor b-AP15 Is Enriched in Cells and Elicits Rapid Commitment to Cell Death

Xin Wang, William Stafford, Magdalena Mazurkiewicz, Mårten Fryknäs, Slavica Brnjic, Xiaonan Zhang, Joachim Gullbo, Rolf Larsson, Elias S. J. Arnér, Padraig D’Arcy, and Stig Linder

Department of Oncology and Pathology, Cancer Center Karolinska (X.W., M.M., S.B., X.Z., P.D., S.L.), and Division of Biochemistry, Department of Medical Biochemistry and Biophysics (W.S., E.S.J.A.), Karolinska Institute, Stockholm, Sweden; and Division of Clinical Pharmacology, Department of Medical Sciences, Uppsala University, Uppsala, Sweden (M.F., J.G., R.L., S.L.)

Received December 26, 2013; accepted April 8, 2014

ABSTRACT

b-AP15 [(3E,5E)-3,5-bis[(4-nitrophenyl)methylidene]-1-(prop-2-enoyl)piperidin-4-one] is a small molecule inhibitor of the ubiquitin specific peptidase (USP) 14 ubiquitin carboxyl-terminal hydrolase (UCH) L5 deubiquitinases of the 19S proteasome that shows antitumor activity in a number of tumor models, including multiple myeloma. b-AP15 contains an α,β-unsaturated carbonyl unit that is likely to react with intracellular nucleophiles such as cysteine thiolates by Michael addition. We found that binding of b-AP15 to USP14 is partially reversible, and that inhibition of proteasome function is reversible in cells. Despite reversible binding, tumor cells are rapidly committed to apoptosis/cell death after exposure to b-AP15. We show that b-AP15 is rapidly taken up from the medium and enriched in cells. Enrichment provides an explanation of the stronger potency of the compound in cellular assays compared with in vitro biochemical assays. Cellular uptake was impaired by 30-minute pretreatment of cells with low concentrations of N-ethylmaleimide (10 μM), suggesting that enrichment was thiol dependent. We report that in addition to inhibition of deubiquitinases, b-AP15 inhibits the selenoprotein thioredoxin reductase (TrxR). Whereas proteasome inhibition was closely associated with cell death induction, inhibition of TrxR was not. TrxR inhibition is, however, likely to contribute to triggering of oxidative stress observed with b-AP15. Furthermore, we present structure-activity, in vivo pharmacokinetic, and hepatocyte metabolism data for b-AP15. We conclude that the strong enrichment of b-AP15 in cells and a rapid commitment to apoptosis/cell death are factors that likely contribute to the strong antitumor activity of this compound.

Introduction

The ubiquitin proteasome system (UPS) is the major intracellular protein degradation system in eukaryotic cells (Hershko and Ciechanover, 1998). The 26S proteasome complex consists of a 20S core particle that is associated with one or two 19S regulatory particles (19S RPs). The 19S particles bind polyubiquitin-linked polypeptides and present them to the 20S degradative units (Groll et al., 1999). The efficient degradation of ubiquitinated substrates requires both unfolding and removal of polyubiquitin chains. The latter function is mediated by specific deubiquitinases that cleave the isopeptide bonds between the C-terminal carboxyl of ubiquitin (Ub) and the amino group of a lysine residue on an adjacent protein (Hershko and Ciechanover, 1998). Three different deubiquitinases are associated with 19S RPs. The ubiquitin specific peptidase (USP) 14 and ubiquitin carboxyl-terminal hydrolase (UCH) 7/UCHL5 enzymes cleave polyubiquitin chains from the distal ends and are suggested to promote substrate rescue rather than

ABBREVIATIONS: 125I-PGJ2, 125I-prostaglandin J2; 19S RP, 19S regulatory particle; AC17, (1E,6E)-1,7-bis(3,4-dimethoxyphenyl)-4-(4-hydroxy-3-methoxybenzylidene)hepta-1,6-diene-3,5-dione; AMC, 7-amino-4-methylcoumarin; b-AP15, (3E,5E)-3,5-bis[(4-nitrophenyl)methylidene]-1-(prop-2-enoyl)piperidin-4-one; b-AP107, (3E,5E)-3,5-bis[(4-chlorophenyl)methylidene]-1-prop-2-enoylpiperidin-4-one; b-AP113, (3E,5E)-3,5-bis[(4-dimethylaminophenyl)methylidene]-1-prop-2-enoylpiperidin-4-one; DMSO, dimethylsulfoxide; DNTB, 5,5′-dithiobis-2-nitrobenzoic acid; ELISA, enzyme-linked immunosorbent assay; GR, glutathione reductase; Himox-1, heme oxygenase (decycling) 1; K18, keratin 18; LC, liquid chromatography; MG262, Z-Leu-Leu-Leu-B(OH)2; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMS-873, 3-[3-(E)-3,5-bis[(4-fluorophenyl)methylidene]-1-[(prop-2-enoyl)piperidin-4-one; DMSO, dimethylsulfoxide; DNTB, 5,5′-dithiobis-2-nitrobenzoic acid; ELISA, enzyme-linked immunosorbent assay; GR, glutathione reductase; Himox-1, heme oxygenase (decycling) 1; K18, keratin 18; LC, liquid chromatography; MG262, Z-Leu-Leu-Leu-B(OH)2; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMS-873, 3-[3-cyclopentylsulfonyl-5-[(3E)-3,5-bis[(4-sulfonilphenylphenoxy)methyl]-1,2,4-triazol-4-yl]pyridine; PS-341, [1(1E)-3-methyl-1-(4S)-3-phenyl-2-[pyrazin-2-ylcarbonyl]amino]propanoyl]amino]butyl]boronic acid; RTA402, methyl (4aR,6aR,8aR,14aR,14bS)-11-isocyano-2,2,6a,6b,9,9,12a-heptamethyl-10,14-dioxo-1,3,4,5,6,7,8,9,14a,14b-decachloropiperidine-4-carboxylate; siRNA, small interfering RNA; TrxR, thioredoxin reductase; UtVS, ubiquitin vinyl sulphone; UCH, ubiquitin carboxyl-terminal hydrolase; UPS, ubiquitin proteasome system; USP, ubiquitin specific peptidase; VLX1545, (3E,5E)-1-acetyl-3,5-bis[(4-nitrophenyl)methylidene]piperidin-4-one; VLX1547, (3E,5E)-3,5-bis[(4-fluorophenyl)methylidene]piperidin-4-one; VLX1548, (3E,5E)-3,5-bis[(4-fluorophenyl)methylidene]piperidin-4-one; VLX1550, (3E,5E)-3,5-bis[(4-fluorophenyl)methylidene]-1-(prop-2-enoyl)piperidin-4-one; WP1130, (5E)-3-(6-bromopyridin-2-yl)-2-cyano-N-(1-pheny1)butyl)acrylamide; YFP, yellow fluorescent protein.
degradation (Lee et al., 2011). USP14 and UCHL5 are cysteine enzymes that become activated after being associated with the proteasome (Lee et al., 2011). The third deubiquitinase associated with the 19S RP is the Zn\(^{2+}\)-dependent metalloprotease regulatory particle subunit 11/pad one homolog-1. This enzyme cleaves entire polyubiquitin chains from substrates in a process that is tightly coupled to degradation (Verma et al., 2002; Yao and Cohen, 2002).

Inhibition of the proteasome is thought to lead to a disruption of the balance of proliferative and antiproliferative signals in the cell, leading to cell-cycle arrest and induction of apoptosis (Adams, 2001; Chauhan et al., 2005; Caravita et al., 2006). Bortezomib (PS-341) is a selective inhibitor of the 26S proteasome that has shown activity against several malignant cell types and has been approved by the US Food and Drug Administration for the treatment of patients with multiple myeloma and mantle cell lymphoma (Chauhan et al., 2005; Caravita et al., 2006). Proteasome inhibitors are reported to sensitize malignant cells to standard chemotherapy and to attenuate inducible resistance to standard chemotherapeutic agents (reviewed in Voorhees and Orlowski, 2006).

The prostaglandin D\(_2\) derivative 12S-prostaglandin J\(_2\) (12S-PGJ\(_2\)) was reported to inhibit cellular deubiquitinase activity (Mullally et al., 2001; Mullally and Fitzpatrick, 2002). This class of prostaglandins contains \(\alpha,\beta\)-unsaturated carboxyl groups that can form covalent adducts with free thiols in proteins by Michael addition (Suzuki et al., 1997). A number of nonprostanoid compounds containing similar unsaturated dienones were also reported to inhibit deubiquitinase activity (Mullally and Fitzpatrick, 2002; Aleo et al., 2006; Kapuria et al., 2010; D’Arcy et al., 2011; Zhou et al., 2013). Since the majority of cellular deubiquitinases are cysteine proteases, their inhibition by 12S-PGJ\(_2\) and structurally related compounds is logical. However, these different compounds show various selectivities to cellular deubiquitinases (Kapuria et al., 2010; D’Arcy et al., 2011; Zhou et al., 2013). Despite sharing the same element of \(\alpha,\beta\)-unsaturated carboxyl groups, 12S-PGJ\(_2\) and AC17 \([1E,6E]-1,7-bis(3,4-dimethoxyphenyl)-4-(4-hydroxy-3-methoxybenzylidine)hepta-1,6-diene-3,5-dione\) are irreversible deubiquitinase inhibitors (Mullally and Fitzpatrick, 2002), whereas b-AP15 \([3E,5E]-3,5-bis(4-nitrophenyl)methylidine]-1-(prop-2-enyl)piperidin-4-one\) was reported to be a reversible inhibitor (D’Arcy et al., 2011).

The USP14/UCHL5 inhibitor b-AP15 inhibits proteasomal function in cells exposed to this compound. b-AP15 is preferentially cytotoxic to tumor cells and shows antitumor activity in both syngeneic and xenograft tumor models (D’Arcy et al., 2011; Tian et al., 2014). The cellular response to b-AP15 is similar, but not identical to, the proteasome inhibitors MG262 [Z-Leu-Leu-Leu-B(OH)\(_2\)] and bortezomib (D’Arcy et al., 2011; Brnjic et al., 2013). Similar to bortezomib, b-AP15 causes induction of chaperone expression and oxidative stress, but these responses are stronger in cells exposed to b-AP15 (Brnjic et al., 2013). The mechanism of the strong oxidative stress response is not known, and is speculated to be related to proteotoxicity (Brnjic et al., 2013).

b-AP15 is cytotoxic to myeloma cells that have acquired resistance to bortezomib (Tian et al., 2014) and is a candidate for clinical drug development. We here set out to characterize the molecular pharmacology of this drug. We show that b-AP15 is actively enriched in target cells and elicits an irreversible commitment to apoptosis/cell death. In addition, we provide a mechanism for the previously reported strong induction of oxidative stress by this compound (Brnjic et al., 2013) by demonstrating that b-AP15 also inhibits the activity of thioredoxin reductase (TrxR). The possible importance of this off-target activity for proteasome inhibition and cell death was investigated.

**Materials and Methods**

b-AP15 was obtained from OnTarget Chemistry AB (Uppsala, Sweden) and bortezomib was obtained from ApoEx (Bromma, Sweden). Compounds were dissolved in dimethylsulfoxide (DMSO). Control cultures received the same final concentration of solvent as treated ones. Compounds b-AP107 \([3E,5E]-3,5-bis(4-chlorophenyl)methylidine]-1-prop-2-enylpiperidin-4-one\), NSC687849 and b-AP113 \([3E,5E]-3,5-bis(4-dimethylaminophenyl)methylidine]-1-prop-2-enylpiperidin-4-one\); NSC687853 were obtained from the National Cancer Institute Developmental Therapeutics Program (Bethesda, MD; http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp). Compounds VLX1545 \([3E,5E]-1-acyetyl-3,5-bis(4-nitrophenyl)methylidine]-piperidin-4-one\), VLX1547 \([3E,5E]-3,5-bis(4-fluorophenyl)methylidine]-piperidin-4-one\), VLX1548 \([3E,5E]-3,5-bis(4-fluoro-3-hydroxphenyl)methylidine]-piperidin-4-one\), VLX1550 \([3E,5E]-3,5-bis(4-fluorophenyl)methylidine]-1-(prop-2-enyl)piperidin-4-one\), and VLX1554 \([3E,5E]-3,5-bis(2-hydroxy-4-nitrophenyl)methylidine]-1-(prop-2-enyl)piperidin-4-one\) were synthesized by OnTarget Chemistry AB. Ubiquitin vinyl sulfone (UVS) was obtained from Boston Biochem (Cambridge, MA).

**Cell Culture.** HCT116 colon carcinoma cells were maintained in McCoy’s 5A modified medium/10% fetal calf serum. A549 cells, HeLa cells, and the proteasome reporter cell line MelJuSo Uh-yellow fluorescent protein (YFP) (Menéndez-Benito et al., 2005) were cultured in Dulbecco’s modified Eagle’s medium/10% fetal calf serum. All cells were maintained at 37°C in 5% CO\(_2\) and were subcultured by trypsinization.

**Cell Viability Assay.** Cell viability was monitored by either the fluorometric microculture cytotoxicity assay or the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. For the fluorometric microculture cytotoxicity assay, tumor cells (5000 cells per well) were seeded in the drug-prepared 384-well plates. Three columns without drugs served as controls and one column with medium only served as the blank. The plates were incubated at 37°C for 72 hours and cell viability was then analyzed by measurement of fluorescence from viable cells after 40-minute incubation with fluorescein by cells with intact plasma membranes (Lindhagen et al., 2008). Cell survival, expressed as the survival index, is defined as fluorescence in test wells divided by fluorescence of control wells, with blank values subtracted, \(x\) 100. For the MTT assay, cells were seeded into 96-well flat-bottomed plates overnight and exposed to drugs as described, using DMSO as the control. At the end of incubations, 10 \(\mu\)l of a stock solution of 5 mg/ml MTT was added into each well, and the plates were incubated 4 hours at 37°C. Formazan crystals were dissolved with 100 \(\mu\)l 10% SDS/10 mM HCl solution overnight at 37°C. Absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Labsystems Multiscan RC; Artisan Technology Group, Champaign, IL) at 590 nm.

**Assessment of Apoptosis.** HCT116 cells were seeded in 96-well microtiter plates at 10,000 cells per well and incubated overnight. Drugs were then added and cells further incubated. At the end of the incubation period, NP40 was added to the tissue culture medium to 0.1% and 25 \(\mu\)l of the content of each well was assayed using the M30-CytoDeath ELISA (Peviva/VLBio AB, Sundbyberg, Sweden) (Hägg et al., 2002). This ELISA is based on a specific antibody against a neoeptope of keratin 18 (K18) that is generated by the action of...
Small Interfering RNA Knockdown Experiments. HeLa cells were maintained in Dulbecco's modified Eagle's high glucose medium with 10% fetal calf serum. For small interfering RNA (siRNA) knockdown, cells were transfected with 0.2 μM siRNA (Thermo Scientific, Wallarth, MA) against USP14, UCHL5, and enhanced green fluorescent protein as a control sequence. Cells were harvested 48 hours after transfection.

Western Blot Analysis. Cell extract proteins were resolved by Tris-acetate PAGE gels (Invitrogen, Carlsbad, CA) and transferred onto a polyvinylidene difluoride membrane for Western blotting. Antibodies were obtained from the following sources: anti-USP14 (A300-919A; Bethyl Laboratories, Montgomery, TX), green fluorescent protein (2555; Cell Signaling Technology, Danvers, MA), anti-ubiquitin keratin 48 (Apu2; Millipore, Billerica, MA), anti-p21(sc-756; Santa Cruz Biotechnology, Santa Cruz, CA), heme oxygenase (decycling) (1 HMox-1; 61072), anti-poly(ADP-ribose) polymerase (C2-10), anti–active caspase-3 (C92-605), and anti–β-actin (AC-15) (all from BD Biosciences, San Jose, CA). Blots were then developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Analysis of Peroxiredoxin Oxidation. HCT116 cells were plated in 10% fetal bovine serum McCoy's 5A supplemented with 25 nM selenium overnight (105 cells per six-well plate). After exposure to compounds for the times indicated, cells were washed with degassed phosphate-buffered saline and incubated for 15 minutes with 180 μl peroxide/oxidation inhibition mixture consisting of degassed 50 mM Tris pH 7.4, 5.0 mM NaCl, 1 mM EDTA, 80 mM methyl methanethiosulfonate (23011; Thermo Scientific), 100 U/ml catalase, and complete EDA-Free Protease Inhibitor Cocktail (1187358001; Roche, Indianapolis, IN). Cells were lysed with the addition of 20 μl 10% NP40 detergent solution (28324; Thermo Scientific) on ice for 15 minutes. Cell debris was cleared via centrifugation for 30 minutes at 17,000g, at 4°C, and 20 μg protein was electrophoresed on 4–20% SDS-PAGE gels under nonreducing conditions and transferred to a nitrocellulose membrane for Western blotting. The antibodies used were Prx1 (LF-PA0086), Prx2 (LF-MA0368), and Prx3 (LF-MA0044; all from AbFrontier, Seoul, Korea).

Live-Cell Analysis of UPS Activity. MelJuSo Ub17857-YFP cells (Menéndez-Benito et al., 2005) were plated in black optically clear-bottom ViewPlates (PerkinElmer, Waltham, MA) overnight and then exposed to b-AP15 or zebrafish. Treatment with compounds that block UPS leads to accumulation of YFP in these cells, and the generated fluorescence was continuously detected in an IncuCyte FLR instrument (Essen BioScience Inc., Ann Arbor, MI).

UbVS Labeling. HCT116 cells were lysed by freeze-thawing 3 x in labeling buffer (50 mM HEPES pH 7.4, 250 mM sucrose, 10 mM MgCl2, 2 mM ATP, 1 mM dithiothreitol). After removal of cell nuclei by centrifugation, total protein (25 μg) was treated with 0, 2.5, 5, 10, 25, and 50 μM b-AP15 for 15 minutes at 37°C and labeled with UbVS (1 μM) for 30 minutes at 37°C. Protein concentration was determined using the Bradford assay (500-0006; Bio-Rad Laboratories, Hercules, CA). Samples were resolved using SDS-PAGE and subjected to immunoblotting using anti-USP14. Alternatively, total protein extracts (25 μg) were incubated for 15 minutes at 37°C in labeling buffer in the presence or absence of b-AP15. Extracts were diluted 1:20 in labeling buffer containing UbVS (1 μM) or b-AP15 and incubation continued at 37°C for 15 minutes. Samples were then subjected to immunoblotting.

Ub-7-Amino-4-Methylcoumarin Assay. We pretreated 195 RPs (2 nM) with DMSO or b-AP15 (6.25, 12.5, 25, or 50 μM) for 2 minutes in assay buffer (25 mM HEPES, 10 mM MgCl2, 2 mM ATP, and 1 mM dithiothreitol) before the addition of 1 mM Ub-AMC (7-aminomethylcoumarin). AMC fluorescence was monitored at 460 nm using a Tecan Infinite 200 instrument (Tecan Group Ltd., Mannédorf, Switzerland).

Retention and Uptake of b-AP15 in Cells. The incorporation of [14C]bAP15 was monitored using LigandTracer White according to the manufacturer's instructions (Ridgeview Instruments AB, Uppsala, Sweden). Briefly, MelJuSo Ub17857-YFP cells were seeded in a tilted dish to achieve one target area and one cell-free reference area. The dishes were kept tilted in an incubator for 4–6 hours, until the cells had adhered. Dishes were then kept horizontally in 10 ml medium to allow the cells to attach firmly to the dish surface for at least 24 hours. In order for the instrument to reach thermal equilibrium, the Ligand-Tracer White instrument was placed in an incubator (37°C in 5% CO2) before the measurements started. Culture dishes containing 5 ml cell culture medium and 4 μM [14C]-bAP15 were placed on the cell dish holder in LigandTracer White and binding of radioactivity to cell-containing areas and reference areas was recorded.

Terminal Deoxynucleotidyl Transferase–Mediated Digoxigenin-Deoxyuridine Nick-End Labeling Assay. Apoptotic cells were determined using the APO-bromodeoxyuridine terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling assay (Invitrogen) according to the manufacturer's instructions. In brief, 5 x 105 cells/ml of cells were seeded in six-well plates and then exposed to drugs as described using DMSO as the control for 24 hours. Cells were harvested and fixed in 1% (v/v) paraformaldehyde and 70% (v/v) ethanol, and were then incubated in the DNA-labeling solution for 60 minutes at 37°C. This was followed by staining with Alexa Fluor 488 dye-labeled anti-bromodeoxyuridine antibody and propidium iodide/RNase A solution for 30 minutes at room temperature. The samples were analyzed by a BD LSRII flow cytometer (BD Biosciences).

Pharmacokinetic Studies. Female NMRI mice (20 g) were used. b-AP15 was dissolved in PEG400/Cremophor EL 1 + 1 and diluted 1:10 in saline. The solution was injected in the tail veins of mice (0.4 mg/ml, 2.8 mg/kg). Blood was collected either after 2 minutes or after 5 and 15 minutes (first sample, vena saphena; terminal sample, orbital plexus). The Stockholm Animal Experimental Ethics Committee North approved this study (N476/11). The experiment was performed by Adlebo AG (Uppsala, Sweden; record AB-13-34). Plasma samples were thawed at room temperature, mixed with a 20-fold volume of acetone-nitrite (containing 200 ng/ml diclofenac as internal standard), shaken, and centrifuged for 10 minutes at 13,000g (Heraeus Pico 17 centrifuge; Thermo Scientific), after which supernatants were pipetted to glass vials to wait for the analysis. Standard samples were spiked at 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 nM concentrations in mouse plasma and were otherwise treated as the study samples. Analysis was performed using liquid chromatography (LC)/mass spectrometry (MS) using an Waters Acuity UPLC system plus a Waters Quattro Ultima triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). The analysis was performed using PerkinElmer control (Oulu, Finland).

Hepatocyte Metabolism. Pooled cryopreserved human hepatocytes (10-donor mix) and pooled cryopreserved CD-1 mouse hepatocytes (16-donor mix) were obtained from Celsis IVT (product numbers X008001 and M005052; Chicago, IL). Samples were analyzed by LC/MS using a Waters Acuity UPLC system plus Waters Xevo G2 quadrupole time-of-flight MS and a Waters Acuity C18 (2.1 x 50 mm, 1.7 μm) column with guard filter. Ion chromatograms were extracted from the time-of-flight MS total ion chromatograms using calculated monoisotopic accurate masses (calculated using Waters Masslynx software for deprotonated molecule) with a 20-mDa window. The metabolites were mined from the data acquired from the last time point, using software-aided data processing (Metabolynx XS including structure-intelligent dealkylation tool and mass defect filter) with manual confirmation. Structures of the observed metabolites were tentatively identified using obtained accurate mass and fragment ion data. These studies were performed by Admescope investigator, Ari Tolonen; laboratory technicians, Birgitta Paldanius and Pirko Hovenae.

Enzyme Activity Assays. Recombinant mammalian TrxR1 activity was determined using a DTNB (5,5'-dithiobis-2-nitrobenzoic acid)-based colorimetric assay (Arnér and Holmgren, 2001). In a 96-well plate, 15 nM TrxR1 was incubated in the presence of 250 μM NADPH (Applichem Inc., St. Louis, MO), 0.1 mg/ml bovine serum albumin, and compounds (1% DMSO) of interest at room temperature for 15 minutes in TE buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA).
Fig. 1. b-AP15 inhibits deubiquitinase activity and proteasome function. (A) Knockdown of USP14 and UCHL5 leads to accumulation of polyubiquitinated proteins in HCT116 cells. Cells were transfected with siRNA overnight, incubated for a further 48 hours, and processed for Western blotting. HCT116 is a colon carcinoma cell line previously found to be sensitive to b-AP15 (D’Arcy et al., 2011). Note that single siRNAs did not induce polyubiquitin accumulation, nor did scrambled siRNAs (not shown). (B) Molecular structure of b-AP15 (NSC687852) and various analogs. The α,β-unsaturated carbonyls are marked with blue filled circles and the Michael acceptor at the acrylamide is denoted with a green circle. The asterisk marks the position of the [14C] in the labeled b-AP15 used in Fig. 4. (C) Dose-response determinations to b-AP15 and various analogs. HCT116 cell viability was determined after 72 hours of exposure using a FMCA. The results are expressed as the percentage of the untreated control and are presented as mean values ± standard deviation. (D) We pretreated 19S RPs (2 nM) with DMSO or b-AP15 (6.25, 12.5, 25, or 50 μM) for 2 minutes in assay.
After incubation, 2.5 mM DTNB (in EtOH) was added to each well and reduction of DTNB to TNB⁻ was followed spectrophotometrically at 412 nm using a VersaMax plate reader. Glutathione reductase (GR) (359960; Calbiochem, Billerica, MA) activity was determined in a 96-well plate, with a reaction mixture of 2 nM GR incubated in the presence of 250 μM NADPH, 0.1 mg/ml bovine serum albumin, and compounds of interest at room temperature for 15 minutes in TE buffer. After incubation, 1 mM glutathione disulfide was added to each well and NADPH consumption was followed spectrophotometrically at 340 nm.

**Cellular TrxR1 Activity.** Cellular TrxR1 activity assays were adapted from Erksson et al. (2009). We plated 1.2 × 10⁶ HCT116 cells plated in a six-well dish in the presence of 25 nM selenium selenite. After overnight incubation, cells were exposed to b-AP15 (in 0.1% DMSO) for 3 hours. Cells were harvested via trypsinization, suspended in medium containing 10% fetal bovine serum, and centrifuged at 800g for 5 minutes and washed 2 × with phosphate-buffered saline. Cell pellets were suspended in 200 μl lysis buffer consisting of 50 mM Tris-HCl pH 7.5, 2 mM EDTA, Protease Inhibitor Cocktail (1187358001; Roche), and 1% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO). Cells were frozen and thawed 3 × using liquid nitrogen followed by 15,000g centrifugation at 4°C for 30 minutes. The supernatant was then collected and the protein concentration was determined using the Bradford assay. We incubated 5 μg lysate in 50 mM Tris-HCl pH 7.5 in the presence of 10 μM Trx1, 275 μM insulin, 1.3 mM NADPH, and 25 mM EDTA for 45 minutes at 37°C in a final volume of 50 μl. Samples were then treated with the reaction mixture. Quantification of the bands indicated an IC₅₀ value of approximately 7 μM. By contrast, b-AP15 did not inhibit the activity of TrxR1 in these experiments. Inhibition of cellular TrxR1 activity was examined using a subline of MelJuSo human melanoma cell line, which expresses cellular proteasome activity was examined using a subline of MelJuSo human melanoma cell line, which expresses cellular proteasome activity and lysed. Extracts (25 μg protein) were incubated with the indicated concentrations of b-AP15 and subsequently incubated with UbVS. Samples were subjected to Western blotting using USP14 and UCHL5 antibodies. (G) Accumulation of polyubiquitin and UbG76V-YFP in MelJuSo cells exposed to b-AP15. The UbG76V-YFP fusion protein is degraded by the proteasome under normal conditions. Note that Hmox-1 is induced at concentrations that do not block the proteasome.

**Statistical Analysis.** Statistical analysis (t test, Pearson correlation coefficient, or Spearman’s rank correlation coefficient as indicated) was performed using Prism software for Apple computers (GraphPad Software, Inc., La Jolla, CA).

**Results**

**Deubiquitinase and Proteasome Inhibition by b-AP15 in Biochemical and Cellular Assays.** The deubiquitinases USP14 and UCHL5 are essential for processing of polyubiquitinated proteins by the proteasome. Knockdown of the expression of both of these enzymes in HeLa cells using siRNA resulted in the accumulation of polyubiquitinated proteins (Fig. 1A), consistent with previous findings (Koulich et al., 2008). The small molecule b-AP15 was previously shown to inhibit the activity of USP14 and UCHL5 (D’Arcy et al., 2011). This molecule contains two different types of Michael acceptors: two symmetrically distributed electrophilic α,β-unsaturated carbonyl units (marked by blue symbols in Fig. 1B) and an acrylamide moiety at the central piperidine ring (green symbol in Fig. 1B). The compound VLX1545, in which the acrylamide was substituted for an acetamide, elicited a similar degree of cytotoxicity as b-AP15 on HCT116 cells (Fig. 1, B and C). Introducing electron-donating groups on the side aryls (hydroxyl substituents, VLX1554; Fig. 1B), led to lower reactivity of the α,β-unsaturated carbonyls (i.e., the Michael acceptors) and resulted in a strong decrease in cytotoxicity (Fig. 1, B and C). Similar results were found in another series containing fluorides on the side aryls; compound VLX1547 that had no group coupled to the nitrogen atom in the piperidine ring showed a similar antiproliferative activity as VLX1550 with an acrylamide. The hydroxyl-containing compound VLX1548 showed lower cytotoxic properties. These findings show that the acrylamide is not required for cytotoxicity and identifies the Michael acceptor reactivity of the α,β-unsaturated carbonyl as the pharmacophore regulating biologic activity.

We determined the ability of b-AP15 to inhibit proteasome deubiquitinase activity using Ub-AMC as the substrate (Fig. 1D). An IC₅₀ of 16.8 ± 2.8 μM was observed. We also used the UbVS probe for activity labeling of USP14. UbVS binds irreversibly to active USP14, resulting in the appearance of a slower migrating form of the protein on Western blots (Fig. 1E). The amount of UbVS-labeled USP14 decreased when increasing concentrations of b-AP15 were present in the reaction mixture. Quantification of the bands indicated an IC₅₀ value of approximately 7 μM. By contrast, b-AP15 did not inhibit total deubiquitinase activity in cell extracts or 20S proteasome activity at concentrations of >50 μM (not shown; D’Arcy et al., 2011).

We next examined inhibition of USP14 in HCT116 cells exposed to b-AP15. Cells were exposed to 1 μM drug and extracts were prepared and labeled with UbVS. Inhibition of USP14 and UCHL5 activity was observed under these conditions (Fig. 1F). We consistently found less effective inhibition of UCHL5 in these experiments. Inhibition of cellular proteasome activity was examined using a subline of MelJuSo human melanoma cell line, which expresses a reporter protein (UbG76V-YFP) degraded by the proteasome. Accumulation of UbG76V-YFP and of K48-linked ubiquitin-conjugated proteins was observed at concentrations of 0.5 and 1 μM b-AP15 (Fig. 1G). We also noted induction of Hmox-1 at low concentrations of b-AP15 (Fig. 1G), which should relate to the induction of oxidative stress (see below).

Accumulation of UbG76V-YFP was also examined by time-lapse microscopy (Fig. 1H). The majority of cells that became YFP positive showed a rounded, apoptotic morphology after 18 hours of drug exposure (white arrowheads in Fig. 1H). A subpopulation of cells did not become positive; these cells remained viable over the observation period. We conclude that inhibition of proteasome function in cells occurs at concentrations of b-AP15 that are >10-fold lower than those required for inhibition of deubiquitinase activity in biochemical assays. Furthermore, proteasome inhibition correlates with subsequent cell death.

**Inhibition of the Proteasomal Function by b-AP15 Is Reversible.** Deubiquitinase inhibitors having similar structural elements as b-AP15 were reported to be irreversible buffer before the addition of 1 μM Ub-AMC. Fluorescence was then recorded continuously. (E) Inhibition of UbVS labeling of USP14 by b-AP15. HCT116 cell extracts (25 μg protein) were incubated with the indicated concentrations of b-AP15 and subsequently incubated with UbVS. Samples were subjected to Western blotting using an USP14 antibody. (F) Inhibition of USP14 and UCHL5 in HCT116 cells. HCT116 cells were exposed to 1 μM b-AP15 for 6 hours and lysed. Extracts (25 μg) were labeled with UbVS (25 μM) for 5 minutes at 37°C. Samples were subjected to Western blotting using USP14 and UCHL5 antibodies. (G) Accumulation of polyubiquitin and UbG76V-YFP in MelJuSo cells exposed to b-AP15. The UbG76V-YFP fusion protein is degraded by the proteasome under normal conditions. Note that Hmox-1 is induced at concentrations that do not block the proteasome. (H) Analysis of accumulation of UbG76V-YFP in MelJuSo cells after exposure to 0.5 μM b-AP15. Note that YFP-positive cells die during the period of observation (white arrowheads), whereas cells remaining YFP negative are unaffected. FMCA, fluorometric microculture cytotoxicity assay; GFP, green fluorescent protein.
enzyme inhibitors (Mullally et al., 2001; Zhou et al., 2013). By contrast, we previously reported reversible inhibition of proteasomal deubiquitinase activity by b-AP15 (D’Arcy et al., 2011). We re-examined the question of reversibility of b-AP15 inhibition of the USP14 deubiquitinase. Active USP14 was labeled with UbVS, generating a slower migrating form on SDS-PAGE (Fig. 2A). The addition of 25 μM b-AP15 prior to labeling and during the labeling step inhibited the reaction of UbVS with USP14 (slot 4). The addition of 25 μM b-AP15 prior to labeling followed by 20-fold dilution of the extracts (to 1.25 μM) resulted in reappearance of the slower migrating form (slot 5). These data show that binding of b-AP15 to USP14 is indeed reversible.

We next determined whether inhibition of proteasomal function is also reversible in living cells. MelJuSo UbG76V-YFP cells were exposed to 0.4 μM b-AP15 for 1 hour, followed by medium change and incubation in drug-free medium. As shown in Fig. 2B, high levels of polyubiquitinated proteins were observed after 1-hour treatment (time 0) and 1 hour after washout, but decreased at 4-hour incubation in the absence of drug. The reporter protein UbG76V-YFP and the proteasome substrate p21Cip1 accumulated until 4 hours after washout, and subsequently decreased. Exposure of MelJuSo UbG76V-YFP cells to bortezomib for 1 hour resulted in transient accumulation of polyubiquitinated proteins (Fig. 2C), consistent with the reversibility of this drug (Adams and Kauffman, 2004).

---

Fig. 2. b-AP15 is a reversible inhibitor of USP14 in vitro and of proteasome function in cells. (A) Reversibility of b-AP15 inhibition of USP14. Total HCT116 cell extracts were prepared and first incubated for 15 minutes at 37°C in the presence or absence of 25 μM b-AP15 (prelabeling step). UbVS (1 μM) was added and incubation continued in the presence or absence of b-AP15 for 15 minutes (slots 2 to 3). Extracts were diluted 1:20 in labeling buffer and 1 μM UbVS was added (slot 5) and incubation continued for 15 minutes. After 30 minutes, incubation was terminated and samples were prepared for immunoblotting using an USP14 antibody. Note that b-AP15 inhibits labeling of USP14 when present at 25 μM, but that this effect is reversed after dilution of the extracts to 1.25 μM. (B) Reversibility of proteasome inhibition by b-AP15. MelJuSo-UbG76V-YFP cells were exposed to 0.4 μM b-AP15 for 1 hour, followed by medium change and incubation in drug-free medium for 0, 1, 4, 8, and 24 hours. Note that the levels of polyubiquitin conjugates decrease after removal of b-AP15 and that UbG76V-YFP and p21 levels decrease at 8 hours (interpretation of the 24-hour time point is difficult due to considerable cell death). Furthermore, note that caspase-3 and PARP cleavage is observed 24 hours after transient drug exposure. (C) Reversibility of proteasome inhibition by bortezomib. MelJuSo-UbG76V-YFP cells were exposed to 0.1 μM for 1 hour, washed, and incubated in drug-free medium. The disappearance of polyubiquitin at 24 hours was not due to cell death (see Fig. 3). GFP, green fluorescent protein; PARP, poly(ADP-ribose) polymerase.
b-AP15 Induces Irreversible Commitment to Cell Death. We noticed cleavage of caspase-3 and poly(ADP-ribose) polymerase in cells transiently exposed to b-AP15 and incubated for an additional 23 hours (Fig. 2B). This result suggests that despite reversibility of proteasome inhibition, cells became committed to apoptosis after 1 hour of exposure to b-AP15. To confirm this finding, we exposed HCT116 cells to b-AP15 for different times and determined caspase-cleaved K18 after 24 hours. We indeed found that 1 hour of exposure to b-AP15 induced a similar level of caspase-cleaved product as continuous exposure (Fig. 3A). By contrast, bortezomib required longer exposure times to induce apoptosis (Fig. 3A). Using cell viability as an end point, we found the difference in IC_{50} values between 1- and 24-hour continuous exposure to b-AP15 to be only approximately 0.2 μM at both low and high concentrations (Fig. 3B). By contrast, bortezomib reduced viability by <10% when used at a 400-nM concentration for 1 hour (Fig. 3C).

Uptake and Retention of b-AP15 in Cells. The results presented in Fig. 1 show a discrepancy between the potency of b-AP15 to block deubiquitinase activity in biochemical assays versus assays of proteasomal function in cells. We hypothesized that these results may be explained by effective uptake from the medium and enrichment of b-AP15 in the cellular compartment. To examine how b-AP15 distributes from medium to cells, we added the compound (1 μM) to culture medium (10% fetal calf serum; 10 ml volume) in the presence or absence of cells and determined the concentration after 1-hour incubation at 37°C. A concentration of 1.15 ± 0.005 μM was observed in the culture medium after 1-hour incubation in the absence of cells compared with 0.83 ± 0.03 μM in the presence of cells (P < 0.0001). This corresponds to a loss of 3.2 nmol of b-AP15 from the medium (0.32 μM × 10 ml). Assuming a cell pellet volume of 50 μl (i.e., 0.5% of culture medium volume), this finding leads to an estimation of an >50-fold enrichment of b-AP15 (and a theoretical total intracellular concentration of >50 μM) over 1 hour. Determination of free intracellular b-AP15 concentrations showed concentrations in the low nanomolar range, suggesting that the majority of drug molecules are associated with cellular macromolecules (not shown). The results suggest that b-AP15 does not bind strongly to serum proteins in the medium, but rapidly distributes into cells. Consistent with weak binding to serum proteins, the concentration of fetal calf serum in the cell culture medium did not significantly influence the ability of b-AP15 to induce cell death (Fig. 4A).

We next examined the kinetics of cellular uptake of [14C]b-AP15 using LigandTracer White (Wang and Albertioni, 2010). Using an b-AP15 concentration of 4 μM, we observed a rapid uptake into cells during 30 minutes of incubation (Fig. 4B, black line). After washout of the substance, approximately 40% of the [14C]b-AP15 signal disappeared during 2 hours. After 10 hours, approximately 50% of the radioactivity remained associated with cells (Supplemental Fig. 1). Pretreating cells with 10 μM N-ethylmaleimide for 30 minutes prior to the addition of [14C]b-AP15 resulted in an approximately 80% decrease in binding (Fig. 4B, red line).

The finding that a significant amount of drug remains associated with cells several hours after washout leads to the prediction that at high drug concentrations, a sufficient number of drug molecules will also be available for inhibiting the proteasome after washout. To test this prediction, we examined the reversibility of proteasome function in cells using increasing concentrations of b-AP15. We indeed found that after exposing MelJuSo cells to 0.8 or 1 μM b-AP15 for 1 hour, polyubiquitin remained 8 hours after changing to drug-free medium (Fig. 4C).

Pharmacokinetics and Metabolism. We injected b-AP15 (dissolved in PEG400/Cremophor EL/saline 5:5:90) into NMRI mice and determined drug concentrations in plasma by MS. A half-life of <5 minutes was observed in plasma (Fig. 5A). This rapid clearance of b-AP15 from plasma is consistent with the above-described rapid uptake into cells.

Metabolite profiling in mouse and human hepatocytes was performed using a 3-hour incubation time. A total of 23 metabolites of b-AP15 were observed; human and mouse hepatocytes had 17 and 19 metabolites, respectively. The observed metabolic reactions included several hydroxylation reactions, together with further hydroxylation reactions and/or glucuronide conjugations. In addition, S-glutathione and
$S$-cysteine conjugates after various hydrogenation reactions were observed. As expected, biotransformation sites for hydrogenation reactions were located to the electrophilic $\alpha,\beta$-unsaturated carbonyl units. The different metabolites are shown in Fig. 5B and the relative abundance of each metabolite is shown in Table 1.

**Elevated Induction of Oxidative Stress by b-AP15 Is Partially Dissociated from Proteasome Inhibition.** A hypothetical intracellular concentration of b-AP15 of approximately 50 $\mu$M is consistent with binding to a considerable fraction of intracellular thiols (estimated to be on the order of 1–5 mM; Di Monte et al., 1984). Although proteasomes are abundant in cells (approximately 1% of cellular protein; Tanaka and Ichihara, 1989), it is unlikely that b-AP15 binds exclusively to proteasomal deubiquitinases in cells. Defining additional cellular targets is important for understanding the activity profile of the drug. Bortezomib is known to induce oxidative stress (Ling et al., 2003) and increases the expression of the oxidative stress–associated marker Hmox-1 (Hamamura et al., 2007; Brnjic et al., 2013). As previously reported (Brnjic et al., 2013), b-AP15 induced a stronger increase in Hmox-1 compared with bortezomib (Fig. 6A). During the course of this study, we noticed that Hmox-1 was induced by 0.2 $\mu$M b-AP15 (Fig. 1G), a drug concentration that elicits limited proteasome inhibition and cytotoxicity. This observation raised the possibility that the oxidative
stress response may be at least partially dissociated from proteasome inhibition.

To further examine whether proteasome inhibition and oxidative stress can be dissociated, we examined the response to a number of b-AP15 analogs (Fig. 6; Supplemental Fig. 2). Similar to b-AP15, compounds b-AP107, VLX1545, and VLX1548 also induced Hmox-1 expression at concentrations that did not induce caspase-3 cleavage (Fig. 6A). The analogs b-AP113, VLX1547, and VLX1550 induced little or no accumulation of polyubiquitin and did not induce strong caspase-3 cleavage or terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling positivity at the concentrations used in Fig. 6, A and B. By contrast, these compounds all induced Hmox-1 expression (Fig. 6A). The results show that b-AP15–related compounds induce oxidative stress by a mechanism that is partially distinct from proteasome inhibition, and that oxidative stress is not sufficient to induce apoptosis.

Compound b-AP113 induces Hmox-1 but not apoptosis at concentrations of 10 and 20 μM over 18–24 hours (Fig. 6). To examine whether oxidative stress induction by this compound kills cells by other cell death modes (e.g., necrosis) over longer time periods, we used an IncuCyte instrument (Essen Bioscience Inc.) to follow cell growth and UbG76V-YFP accumulation. At a concentration of 10 μM, b-AP113 did not affect MelJuSo cell viability over 72 hours (Supplemental Fig. 3).

b-AP15 Is an Irreversible Inhibitor of Thioredoxin Reductase. The thioredoxin and glutathione systems are essential for cellular redox-homeostasis. Cyclopentenone prostaglandins were previously found to inhibit activity of TrxR (Moos et al., 2003; Shibata et al., 2003), suggesting to us that b-AP15 may possibly also target TrxR. TrxR enzymatic activity is dependent on the motif Gly-Cys-Sec-Gly, in which Sec is selenocysteine, whereas GR activity is dependent on the two cysteine residues Cys58 and Cys63 (Berkholz et al., 2008). The Sec residue of TrxR is particularly reactive to electrophiles (Rundlöf and Arnér, 2004) and inhibition of TrxR typically induces Hmox-1 expression (Mostert et al., 2003). As shown in Fig. 7A, TrxR activity is inhibited by b-AP15 in
TABLE 1
The relative LC/MS peak areas for b-AP15 and its metabolites in mouse and human hepatocytes
The quantification is based on the assumption of identical LC/MS response between all compounds.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mouse Hepatocytes</th>
<th>Human Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>2.7</td>
<td>5.0</td>
</tr>
<tr>
<td>M1</td>
<td>0.5</td>
<td>3.6</td>
</tr>
<tr>
<td>M2</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>M3</td>
<td>0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>M4</td>
<td>7.8</td>
<td>5.5</td>
</tr>
<tr>
<td>M5</td>
<td>11.4</td>
<td>4.8</td>
</tr>
<tr>
<td>M6</td>
<td>23.2</td>
<td>21.1</td>
</tr>
<tr>
<td>M7</td>
<td>6.5</td>
<td>0.4</td>
</tr>
<tr>
<td>M8</td>
<td>1.1</td>
<td>2.5</td>
</tr>
<tr>
<td>M9</td>
<td>10.0</td>
<td>2.0</td>
</tr>
<tr>
<td>M10</td>
<td>8.6</td>
<td>—</td>
</tr>
<tr>
<td>M11</td>
<td>6.5</td>
<td>—</td>
</tr>
<tr>
<td>M12</td>
<td>6.8</td>
<td>—</td>
</tr>
<tr>
<td>M13</td>
<td>1.3</td>
<td>3.7</td>
</tr>
<tr>
<td>M14</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>M15</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td>M16</td>
<td>5.0</td>
<td>0.7</td>
</tr>
<tr>
<td>M17</td>
<td>3.7</td>
<td>0.6</td>
</tr>
<tr>
<td>M18</td>
<td>—</td>
<td>3.8</td>
</tr>
<tr>
<td>M19</td>
<td>—</td>
<td>1.6</td>
</tr>
<tr>
<td>M20</td>
<td>—</td>
<td>5.4</td>
</tr>
<tr>
<td>M21</td>
<td>—</td>
<td>11.0</td>
</tr>
<tr>
<td>M22</td>
<td>—</td>
<td>4.9</td>
</tr>
<tr>
<td>M23</td>
<td>—</td>
<td>19.2</td>
</tr>
</tbody>
</table>

—, metabolites were not detected in these species.

HCT116 cells exposed to b-AP15. Furthermore, TrxR activity was inhibited by b-AP15 in an in vitro assay at a concentration of approximately 1 μM (Fig. 7B). By contrast, GR activity was not significantly inhibited. Compounds b-AP107, b-AP113, VLX1545, VLX1547, and VLX1550 also inhibited TrxR1 activity with somewhat different potencies (Fig. 7B), whereas compounds VLX1548 and VLX1554 did not inhibit TrxR activity at the concentrations tested. Bortezomib did not inhibit TrxR or GR activity (Fig. 7B). We conclude that b-AP15 and some of its analogs inhibit TrxR and induce Hmox-1 expression. Furthermore, the comparatively low ability of VLX1554 to induce Hmox-1 (Fig. 6) correlated with weak TrxR inhibition or Hmox-1 induction are events that are not necessarily linked to impaired proteasome function.

A TrxR-related protein was found to be associated with the proteasome (Wiseman et al., 2009). We therefore examined whether TrxR inhibition influences proteasome function in tumor cells. As shown in Fig. 7F, auranofin did not induce a similar degree of polyubiquitin accumulation in HCT116, A549, or MelJuSo cells as b-AP15. Furthermore, auranofin did not induce UbO76-YFP accumulation in MelJuSo cells. Auranofin consistently induced higher levels of Hmox-1 compared with b-AP15 in these different cell lines. A549 cells show constitutive Nrf2 activity, resulting in baseline Hmox-1 expression. b-AP15 and auranofin induced dimerization of periredoxin-3, showing oxidative stress (Poynton and Hampton, 2014), whereas bortezomib did not (Supplemental Fig. 4). We conclude that TrxR inhibition or Hmox-1 induction are events that are not necessarily linked to impaired proteasome function.

Discussion

Previous work demonstrated that the small molecule b-AP15 inhibits USP14/UCHL5 deubiquitinase activity and shows promising antitumor activity in syngeneic and xenograft tumor models (D’Arcy et al., 2011). b-AP15 induces accumulation of high molecular mass polyubiquitin in the submicromolar range. By contrast, inhibition of deubiquitinasase activity in vitro is observed at concentrations of approximately 10 μM. Here we provide an explanation for this apparent discrepancy by showing that b-AP15 is strongly enriched in cells. Enrichment appears to be driven by thioldependent mechanisms, as evidenced by inhibition of uptake by short pretreatment of cells with low concentrations of N-ethylmaleimide. Concentrations of free b-AP15 in cells were low (1–5 nM), consistent with intracellular covalent binding of the compound to thiols. In contrast with the rapid sequestration to intracellular thiols, free b-AP15 was recovered from cell culture medium containing fetal calf serum, showing that the compound did not irreversibly bind serum proteins. Furthermore, increasing the fetal calf serum concentration in the culture medium did not significantly decrease the cytotoxic activity of b-AP15. Our findings suggest that b-AP15 is rapidly distributed from serum/plasma to cellular compartments both in cell cultures and in exposed mice.

b-AP15 belongs to a class of compounds that contain α,β-unsaturated carbonyl groups and that inhibit cellular deubiquitinases. The compound also contains a potentially reactive acrylamide residue, but this group was not necessary for cytotoxic activity in colon cancer cells and was not found to be modified in hepatocytes. We synthesized a number of compounds in which various groups were coupled to the α,β-unsaturated carbonyls. These compounds show cytotoxic activities in the 5–10 μM range (unpublished data) and we assume that the side groups are released. The side aryls of b-AP15 are substituted with electron withdrawing –NO2 groups that increase the Michael acceptor capability of the α,β-unsaturated carbonyls. As expected, introduction
of electron-donating groups on the side aryls (b-AP113, VLX1554, and VLX1558) significantly impaired the proteasome inhibiting and cytotoxic activity of these compounds, presumably by decreasing the reactivity of the $\alpha,\beta$-unsaturated carbonyls. Other compounds containing similar structural elements as b-AP15 were reported to show different specificities with regard to deubiquitinase inhibition. $\alpha$-PGJ2 was reported to inhibit UCHL1 and UCHL3 (Li et al., 2004) and WP1130 [(S,E)-3-(6-bromopyridin-2-yl)-2-cyano-N-(1-phenylbutyl)acrylamide] inhibits USP9x, USP5, USP14, and UCHL5 (Kapuria et al., 2010). Similar to b-AP15, the compound AC17 was found to specifically inhibit proteasomal deubiquitinases (Zhou et al., 2013). The mechanisms underlying these different specificities are unclear, but presumably reflect the accessibility of cysteines in the active sites of different deubiquitinases to these small molecules. It remains to be explained how both USP and UCH classes of deubiquitinases, which show limited homology (Komander et al., 2009), can be inhibited by these compounds. We generally find more effective inhibition of USP14 compared with UCHL5 both in cell extracts and using 19S proteasomes (data not shown), raising the possibility that USP14 is the most high affinity target for b-AP15.

Target residence time is known to be affected by the context of the reactive electrophile (Serafimova et al., 2012). Compounds having similar electrophiles as b-AP15 (i.e., $\alpha,\beta$-unsaturated carbonyl chains) were reported to be irreversible deubiquitinase inhibitors (Mullally and Fitzpatrick, 2002; Zhou et al., 2013). Cyclopentenone prostaglandins containing Michael acceptors bind soluble cysteine thiols, such as cysteine and glutathione, in a reversible manner, whereas the solid state Michael reaction is less reversible, possibly due to decreased molecular motion (Suzuki et al., 1997). Binding of b-AP15 to USP14 is reversible (Fig. 2), consistent with our previous results (D’Arcy et al., 2011). The $\alpha,\beta$-unsaturated carbonyl conjugated system in b-AP15 is apparently prone to retro-Michael reactions under some conditions. At low concentrations (0.4 and 0.6 $\mu$M), transient exposure to b-AP15 leads to the disappearance of polyubiquitin after 8 hours of incubation in drug-free medium (Figs. 2 and 4). Interestingly, cells nevertheless become committed to apoptosis/cell death (Figs. 2 and 3). We do not presently understand the mechanism of this rapid and seemingly faithful commitment. At higher concentrations (0.8 and 1 $\mu$M), polyubiquitin conjugates did not disappear in transiently exposed cells. We interpret this result to suggest that a sufficient number of drug molecules are present in cells to be able to continuously inhibit proteasomal deubiquitinases. The slow release of the compound from cells (Fig. 4B) is consistent with this assumption. In contrast with the rapid commitment to cell death by b-AP15, transient exposure to bortezomib did not result in apoptosis/cell death (Fig. 3C). Promising in vivo activities of b-AP15 were observed in a number of tumor models (D’Arcy et al., 2011; Tian et al., 2014), and this activity may partially be due to the ability of the compound to induce cell death after relatively short periods of exposure.
Fig. 7. Inhibition of TrxR by b-API15 cannot explain apoptosis induction or proteasome inhibition. (A) TrxR enzyme activity was determined in HCT116 cells exposed to the indicated concentrations of b-API15 for 3 hours. (B) TrxR and GR enzyme activity was determined in the presence of the indicated compounds. Note that b-API15 and its analogs inhibit TrxR (♦), but not GR (■), enzyme activity. (C) Irreversible inhibition of TrxR by b-API15. b-API15 was added to purified TrxR and enzyme activity was determined before or after gel filtration. (D) Accumulation of caspase-cleaved K18 was determined in cell cultures after 14 hours of exposure of HCT116 cells to auranofin (1.5 μM), bortezomib (0.1 μM), and/or b-API15 (1 μM) as indicated. ELISA was used to determine caspase-cleaved K18. (E) Accumulation of caspase-cleaved K18 was determined in cell cultures after 14 hours of exposure of HCT116-BCL2 cells to different drugs. Conditions are the same as in D. (F) Auranofin does not block proteasome function. HCT116, A549, or MelJuSo UbG76V-YFP cells were exposed to b-API15 (1 μM) or auranofin (1.5 μM) for 6 hours and extracts were analyzed by Western blotting. Note that whereas auranofin induces strong Hmox-1 expression, accumulation of polyubiquitin and Ub-YFP is insignificant compared with b-API15. A549 cells are known to constitutively express Nrf2, leading to baseline Hmox-1 expression in the cell line. BCL2, B-cell lymphoma 2; BZ, bortezomib; veh, vehicle.
Michael acceptors are traditionally avoided by drug developers due to their reactivity. However, many biologically relevant pathways are targeted by thiol-reactive compounds and covalent coupling to thiols is a potentially important mechanism of bioactivity (Amslinger, 2010). An irreversible inhibitor of the AAA family ATPase VCP (p97/Cdc48p) was recently described (Magnaghi et al., 2013). This compound (NMS-873; 3-[3-cyclopentylsulfonyl-5-[3-methyl-4-(4-methylsulfonylphenyl) phenoxy]methyl]-1,2,4-triazol-4-yl]pyridine) covalently modifies an active site cysteine of this enzyme. Similar to b-AP15, NMS-873 induced accumulation of polyubiquitin and cancer cell death. The homo-triterpenoid bardoxolone methyl (RTA402; methyl 43aS,6aR,8aS,8aR,12aR,14aR,14bS)-11-isocyano-2,2,6a,6b,9,9,12a-heptamethyl-10,14-dioxo-1,3,4,5,6,7,8a,14a,14b-decahydropicene-4a-carboxylate) contains an α,β-unsaturated carbonyl unit (Liby et al., 2007) and is in clinical development for the treatment of diabetes-associated chronic kidney disease (Pergola et al., 2011). Bardoxolone methyl has also been given orphan drug status by the US Food and Drug Administration for the treatment of pancreatic cancer (http://www.news-medical.net/news/2008/10/16/42030.aspx).

We here report that in addition to inhibition of proteasome function, b-AP15 inhibits TrxR. The active site of this enzyme contains a selenocysteine residue that is reactive to electrophiles (Rundlöf and Arner, 2004) and TrxR inhibition by b-AP15 is therefore not surprising. In contrast with inhibition of USP14, inhibition of TrxR was found to be irreversible, possibly reflecting the strong reactivity of the drug to the enzyme. By contrast, GR enzyme activity was not inhibited. GR activity is dependent on two cysteine residues (Cys58 and Cys63) (Berkholz et al., 2008). The TrxR over GR selectivity of b-AP15 shows specificity for some cysteines, and is consistent with previous reports that electrophilic compounds selectively react with cellular cysteines (Dennehy et al., 2006; Liebler, 2008) and the fact that TrxR, with its selenocysteine residue, is particularly prone to inhibition by electrophiles, including prostaglandin derivatives (Moos et al., 2003). The bioactive cyclopentenone 15β-prostaglandin J2, known to induce cellular oxidative stress, has been shown to inhibit TrxR activity (Shibata et al., 2003). Contrary to expectation, 15β-prostaglandin J2 was reported to bind Cys55 or Cys69, and not Sec, in TrxR (Shibata et al., 2003). We do not presently know which cysteine residue in TrxR is targeted by b-AP15. We previously reported that oxidative stress is an important mediator of apoptosis induction by b-AP15 (Brnjic et al., 2013), and it is likely that TrxR inhibition contributes to apoptotic signaling. Since bortezomib induces oxidative stress in the absence of TrxR inhibition, we hypothesize that the strong oxidative stress observed in cells exposed to b-AP15 is due to both proteasome and TrxR inhibition. Our experiments combining bortezomib and auranofin suggested that TrxR inhibition contributes to the apoptotic activity of b-AP15, but is of limited importance for the superior cytotoxicity of b-AP15 over bortezomib. We also found that TrxR inhibition is unlikely to contribute to proteasome inhibition by b-AP15.

This was important to establish in the light of reports showing that thioredoxin-related protein of 32 kDa (thioredoxin-like 1) is a subunit of the 26S proteasome and that auranofin treatment changes the expression of proteasomal proteins (Guidi et al., 2012).

This study clarifies a number of questions with regard to the mechanism of action and pharmacology of the deubiquitinase inhibitor b-AP15. The findings of rapid uptake and enrichment in cells and the commitment to apoptosis/cell death within 1 hour lead to some optimism with regard to clinical activity of b-AP15 (or optimized leads of this compound).

Acknowledgments

The authors thank Admescope for performing LC/MS pharmaco-kinetic studies and metabolism studies, as well as OnTarget Chemistry (Uppsala, Sweden) for synthesizing compounds. The authors also thank Hans Rosén (Vivolux AB) for providing support and input at various levels as well as organizational help, Adlego AB for performing pharmaco kinetic studies, and the National Cancer Institute Developmental Therapeutics Program for supplying compounds. Arne Holmgren kindly provided Trx1. Finally, the authors thank Maria Rydäker, Lena Lenhammar, and Christina Leek for excellent technical support.

Authorship Contributions

Participated in research design: Wang, Stafford, Fryknäs, Brnjic, Gullbo, Larsson, Arner, D’Arcy, Lindner.

Conducted experiments: Wang, Stafford, Mazurkiewicz, Fryknäs, Brnjic, Zhang, D’Arcy.

Performed data analysis: Wang, Stafford, Mazurkiewicz, Fryknäs, Brnjic, Zhang, Gullbo, Larsson, Arner, D’Arcy, Lindner.

Wrote or contributed to the writing of the manuscript: Wang, Stafford, Mazurkiewicz, Fryknäs, Brnjic, Zhang, Gullbo, Larsson, Arner, D’Arcy, Lindner.

References


Commitment to Apoptosis by a 19S Deubiquitinase Inhibitor


Address correspondence to: Stig Linder, Cancer Center Karolinska, Department of Oncology and Pathology, Karolinska Institute, SE-171 76 Stockholm, Sweden. E-mail: Stig.Linder@ki.se
The 19S deubiquitinase inhibitor b-AP15 is enriched in cells and elicits rapid commitment to cell death

Xin Wang, William Stafford, Magdalena Mazurkiewicz, Mårten Fryknäs, Slavica Brjnic, Xiaonan Zhang, Joachim Gullbo, Rolf Larsson, Elias Arnér, Padraig D'Arcy and Stig Linder

Supplementary Figure 1. Uptake of b-AP15 into cells.

Uptake of [14C] b-AP15 over 12 hours was determined using LigandTracer® White. Association of radioactive drug with cells was determined in real-time.
Supplementary Figure 2. Structures of b-AP15 (NSC687852) and the analogues b-AP107 (NSC687449) and b-AP113 (NSC687853).

All compounds contain an \( \alpha, \beta \)-unsaturated carbonyl units (blue dots) and an acrylamide moiety with a potential Michael acceptor (green dot). Note that the cytotoxic activity of the compounds correlates with the reactivity of the \( \alpha, \beta \)-unsaturated carbonyl units (whereas compound b-AP113, which shows weak cytotoxic activity, contains the acrylamide).
**Supplementary Figure 3.** Response of MelJuSo Ub^{G76V}-YFP cells to b-AP15 and b-AP113.

MelJuSo Ub^{G76V}-YFP cells were monitored after exposure to the indicated compounds. Photographs were taken at the indicated time-points using an IncuCyte instrument.
MelJuSo Ub^{G76V}-YFP cells were monitored after exposure to the indicated compounds. Photographs were taken at the indicated time-points using an IncuCyte instrument.
Supplementary Figure 4. Analysis of oxidative stress induction by b-AP15 and the thioredoxin reductase inhibitor auranofin.

Analysis of monomeric and dimeric forms of periredoxin 3 (Prx-3) as an indicator of oxidative stress. HCT116 cells were exposed to the indicated compounds for 45 minutes. Lysates were prepared as described in Methods and electrophoresed under non-reducing conditions. Note the increase in the oxidized form of Prx-3 (localized to the mitochondrial matrix) after exposure to auranofin and b-AP15. Both bands labelled as oxidized forms disappear with treatment with DTT. For details on the methodology, see Poynton and Hampton, Periredoxins as biomarkers of oxidative stress, Biochim Biophys Acta 1840 (2014) 906.