Bcr-Abl ubiquitination and Usp9x inhibition block kinase signaling and promote CML cell apoptosis


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Although chronic myelogenous leukemia (CML) is effectively controlled by Bcr-Abl kinase inhibitors, resistance to inhibitors, progressive disease, and incomplete eradication of Bcr-Abl-expressing cells are concerns for the long-term control and suppression of this disease. We describe a novel approach to targeting key proteins in CML cells with a ubiquitin-cycle inhibitor, WP1130. Bcr-Abl is rapidly modified with K63-linked ubiquitin polymers in WP1130-treated CML cells, resulting in its accumulation in aggresomes, where it is unable to conduct signal transduction. Induction of apoptosis because of aggresomal compartmentalization of Bcr-Abl was observed in both imatinib-sensitive and -resistant cells. WP1130, but not Bcr-Abl kinase inhibitors, directly inhibits Usp9x deubiquitinase activity, resulting in the down-regulation of the prosurvival protein Mcl-1 and facilitating apoptosis. These results demonstrate that ubiquitin-cycle inhibition represents a novel and effective approach to blocking Bcr-Abl kinase signaling and reducing Mcl-1 levels to engage CML cell apoptosis. This approach may be a therapeutic option for kinase inhibitor–resistant CML patients. (Blood. 2011;117(11):3151-3162)

Introduction

Chronic myelogenous leukemia (CML) is associated with a chromosomal abnormality in the hematopoietic stem cell that results in the expression of Bcr-Abl with unregulated tyrosine kinase activity. These observations supported the development and clinical testing of the first Bcr-Abl kinase inhibitor, imatinib, which demonstrated remarkable clinical efficacy in CML patients. Imatinib is the frontline therapy for CML and other Bcr-Abl–expressing leukemias, and most patients treated with imatinib in the chronic phase achieve a complete cytogenetic response. However, molecular studies of imatinib-treated patients in remission demonstrated that Bcr-Abl expression is still detectable in most cases, and discontinuation of imatinib therapy often results in disease relapse. Limited duration of imatinib response is also common in advanced CML patients, and imatinib resistance can occur at any stage of the disease. Acquired imatinib resistance and disease progression are frequently characterized by Bcr-Abl mutations and posttranslational modifications that affect imatinib binding and kinase inhibition. Some of the molecular changes in imatinib-resistant disease can be overcome with second-generation tyrosine kinase inhibitors, which bind Bcr-Abl with higher affinity or inhibit imatinib-insensitive kinases associated with resistance. However, the activity of these inhibitors can also be limited by mutations and other mechanisms. These observations suggest that additional approaches and targeting strategies may be needed to provide long-term effective treatment for CML patients.

Kinase inhibition by small molecules that bind the ATP or the switch pocket region of Bcr-Abl are effective inhibitors but require continuous treatment because Bcr-Abl protein levels themselves are not directly regulated through kinase inhibition. Some evidence suggests that Bcr-Abl can function as a protein scaffold to organize signaling complexes that are not fully dependent on kinase activity. These observations suggest that compounds that modulate Bcr-Abl protein levels may be more effective and appropriate for CML therapy in some settings. In light of that possibility and as a novel approach to overcoming kinase inhibitor resistance, several compounds have been described that affect Bcr-Abl function through mechanisms other than direct kinase inhibition. These include heat shock protein 90 (Hsp90) inhibitors, arsenic trioxide, homoharringtonine, histone deacetylase inhibitors, proteasome inhibitors, PP2A activators, and others. All are reported to lead to CML cell death through down-regulation of Bcr-Abl expression or a loss of Bcr-Abl stability. However, most of these compounds reduce Bcr-Abl levels only after extended incubation intervals and display only limited selectivity for Bcr-Abl, which may increase their toxicity and decrease their clinical utility. Additional compounds that induce rapid changes in Bcr-Abl levels with limited impact on other proteins are still needed.

In a previous study, we described the discovery and antileukemic activity of WP1130, a small molecule with an unknown mechanism of Bcr-Abl down-regulation. In the present study, we demonstrate that WP1130 rapidly induces ubiquitination of Bcr-Abl, resulting in its relocalization from the cytoplasm into compact, intracellular protein complexes called aggresomes. This modification results in the loss of downstream Bcr-Abl oncogenic activity.
signaling. We further demonstrate that WP1130 directly inhibits Usp9x, a deubiquitinating enzyme (DUB) recently reported to regulate the stability of Mcl-1, an antiapoptotic protein expressed in many tumors, including hematologic malignancies. Mcl-1 is associated with drug resistance and survival in hematopoietic malignancies. WP1130-mediated Up9x inhibition is associated with reduced Mcl-1 levels, and together with blocked Bcr-Abl kinase signaling, results in the rapid onset of apoptosis. These results suggest that targeting specific ubiquitin-cycle regulators may emerge as a novel therapeutic approach to inhibiting oncoprotein signaling and reducing elevated apoptotic thresholds.

Methods

Compounds, chemical reagents, and affinity matrices

WP1130, imatinib, and dasatinib were synthesized and purified by William Bornmann (M. D. Anderson Cancer Center, Houston, TX). TG101209 was synthesized and provided by Dr Hollis Showalter (University of Michigan, Ann Arbor). ABT-263 was purchased from Accela ChemBio. All compounds were made up as 20mM stock solutions (in dimethylsulfoxide (DMSO)), stored frozen at −20°C, and diluted to aqueous medium just before use. Other reagents used in this study were: bortezomib (Millennium Pharmaceuticals); Mini-Complete and PhosSTOP inhibitory cocktails (Roche Applied Science); ubiquitin C-terminal 7-amido-4-methylcoumarin (UA-AMC), and hemagglutinin-tagged ubiquitin vinyl methyl sulfone (HA-UbVs), Suc-LLVY-AMC, Boc-LRR-AMC, MG-132, lactacystin, and 20S human proteasome (Boston Biochem). Affinity matrices of Rap80-agarose beads, ataxin-agarose beads, and purified polyubiquitin chains (K48/K63-linked) were also obtained from Boston Biochem. 17-Azlylamino-17-demethoxygeldenamycin (17-AAG) was purchased from LC Laboratories, made up and stored frozen as a 20mM stock solution.

Cell lines and patient samples

K562, K562R, BV-173, BV-173R, and WDT-2 cell lines were grown and maintained as described previously. BaF3 cells were maintained in the K562, K562R, BV-173, BV-173R, and WDT-2 cell lines were grown and maintained as described previously.22,23 Mcl-1 is associated with drug resistance and survival in hematopoietic malignancies.24 WP1130-mediated Up9x inhibition is associated with reduced Mcl-1 levels, and together with blocked Bcr-Abl kinase signaling, results in the rapid onset of apoptosis. These results suggest that targeting specific ubiquitin-cycle regulators may emerge as a novel therapeutic approach to inhibiting oncoprotein signaling and reducing elevated apoptotic thresholds.

shRNA and Usp9x silencing

BV-173 cells were infected with the lentiviral expression system for short hairpin RNA (shRNA), LVX-shRNA1, and LVX-shRNA2-Usp9x, kindly provided by Dr Dzwokai Ma (University of California, Santa Barbara). In brief, HEK293T cells were transfected with the lentiviral packaging vectors pMD2.G and psPax2 (Addgene) together with the LVX-shRNA vectors to produce virus using PolyFect as described by the manufacturer (QIAGEN). The medium was changed to RPMI 1640 with 20% fetal bovine serum and 10mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), pH 7.4, the following day, and the viral supernatant was collected by centrifugation on day 2 after transfection. BV-173 cells were spin infected for 2 hours with 1.5 µL of viral supernatant containing 8 µg/mL of Polybrene (Sigma-Aldrich) at 2000 at 32°C. Two days after infection, the medium was changed and 1 µg/mL of puromycin was added. After puromycin selection (5 days), viable cells were recovered by Ficoll gradient separation, and Usp9x levels were examined by immunoblotting. Those with stable reduction of Usp9x were used to assess Mcl-1 levels and to analyze apoptotic sensitivity to imatinib and ABT-263.

Lysate preparation, antibodies, and Western blotting

Total cell lysates were prepared by boiling and sonicating cell pellets in 1× Laemmli reducing sample buffer. To prepare detergent-soluble and -insoluble fractions, cells were lysed in cold isotonic lysis buffer (10mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 150mM NaCl, along with MiniComplete and PhosSTOP) for 15 minutes on ice and centrifuged for 10 minutes at 20 000g. The clarified supernatant was used as the detergent-soluble cell fraction. The residual pellet was washed and extracted in Laemmli reducing sample buffer and briefly sonicated to derive the detergent-insoluble fraction. Equal volumes of cellular lysate or equal protein amounts were electrophoresed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes (Whatmann). Proteins were detected by immunoblotting, as described previously.27

Antibodies used in this study were: anti-pY-Stat5, p53, CrkL, anti-poly(adenosine diphosphate)-ribose polymerase (anti-PARP), anti-caspase 3, and anti–Mcl-1 (Cell Signaling Technology); anti-actin (Sigma-Aldrich); polyclonal anti-ABL (K12), monoclonal anti-ABL (SH2 domain; 8E9), anti-ubiquitin clone P3D1, anti-Hsp90, anti-Hsp70, anti-Jak2, anti-CrkL, anti–α-tubulin, and horseradish peroxidase–conjugated goat anti-rabbit/mouse/rat immunoglobulin G (IgG; Santa Cruz Biotechnology); anti-HA clone 3F10 (Roche Applied Science); and anti-Usp9x (Bethyl Laboratories). Caspase 9 antibody was from Calbiochem.

Plasmids and electroporation

The eGFP-coding region was cloned from pLEGFPc by polymerase chain reaction using a 5’ primer with an EcoR1 restriction site, GAATTCCCGC-
proteasome inhibition, cells were treated with WP1130 (5 μM) or MG132 (5 μM) for 2 hours and lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 5 mM EDTA [ethylenediaminetetraacetic acid], 150 mM NaCl, 1% Triton X-100). Lysates were clarified by centrifugation at 20,000 g for 10 minutes, and equal amounts of protein from each sample were incubated at 37°C with 100 μM fluorogenic substrate. To assay for direct inhibition of the 20S proteasome in vitro, purified 20S human proteasome (200 ng) was incubated with WP1130 (5 μM), MG132 (5 μM), or lactacystin (5 μM) for 30 minutes at 37°C before addition of the substrates. Fluorescence intensity was measured using a spectrophotometer at an excitation of 360 nm and an emission of 460 nm. Assays were performed in triplicate, and statistical significance was determined with a paired Student’s t-test.

ROS assay

Leukemic cells (1 × 10⁶) were treated with DMSO, WP1130 (5 μM), or a positive (0.5 mM H₂O₂) or negative (1 mM dithiothreitol) effector of reactive oxygen species (ROS) content for 2 hours at 37°C. Cells were washed and resuspended in PBS containing 1% (w/v) bovine serum albumin and 0.5% (w/v) triton X-100. The cells were fixed in 4% formaldehyde in PBS for 15 minutes at room temperature and then permeabilized in 0.5% Triton X-100 for 5 minutes. The cells were then washed with PBS and blocked with 2% (w/v) BSA in PBS for 30 minutes. The cells were then incubated with the primary antibody for 1 hour at room temperature, followed by washing and incubation with the secondary antibody (Alexa Fluor 488 or Alexa Fluor 546) for 30 minutes. The cells were then washed with PBS and viewed under a confocal microscope.

DUB-labeling assays

To assay for changes in the activity of cellular DUB enzymes, leukemic cells were treated with WP1130 (5 μM) for 2 hours and lysed in DUB buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 150 mM NaCl, and 1 mM phenylmethylsulfonylfluoride. Briefly, 5 μg of clarified lysate from untreated or treated cells was incubated with 500 nM Ub-AMC in a 100-μL reaction volume at 37°C, and the release of AMC fluorescence intensity was measured using a spectrophotometer at an excitation of 380 nm and an emission of 480 nm using a spectrofluorometer.

Results

WP1130 induces protein ubiquitination and Bcr-Abl protein redistribution in both imatinib-sensitive and -resistant cells

We previously reported that WP1130 induced down-regulation of Bcr-Abl in imatinib- and dasatinib-sensitive and -resistant CML cells. To determine the selectivity for Bcr-Abl, several kinases expressed in CML cells were assessed for WP1130-mediated down-regulation. Only Bcr-Abl protein levels were reduced after WP1130 treatment, suggesting specificity for this chimeric protein (Figure 1A). We expressed eGFP-tagged Bcr-Abl (WT) in
BaF3 cells and observed rapid down-regulation of the ectopically expressed Bcr-Abl after WP1130 treatment (Figure 1B). We further established stable BaF3 cell lines expressing W/T and T315I variants of Bcr-Abl and observed high sensitivity to WP1130-mediated apoptosis in both cases. In contrast, BaF3 cells expressing T315I eGFP-Bcr-Abl were imatinib resistant, suggesting that the eGFP tag did not influence Bcr-Abl-mediated cellular transformation or imatinib sensitivity (Figure 1C). We further investigated the effects of WP1130 on eGFP-Bcr-Abl by fluorescent microscopy and observed a rapid accumulation of eGFP-Bcr-Abl into compact, high-density Bcr-Abl clusters after WP1130 incubation (Figure 1D).

Hsp90 inhibition by geldanamycin and its analogs has been reported to affect Bcr-Abl stability and cellular distribution. We compared the antiproliferative effects of Hsp90 inhibitor (17-AAG) with those of WP1130. Both WP1130 and 17-AAG displayed antiproliferative effects in CML lines expressing either W/T Bcr-Abl (BV-173, Figure 2A left) or T315I mutant Bcr-Abl (BV-173R, Figure 2A right), but distinctions in the activation of caspases and onset of apoptosis were observed (Figure 2B). We noted a reduction in Hsp90 and Bcr-Abl association in 17-AAG–treated cells, which can lead to Bcr-Abl down-regulation through ubiquitination, as reported previously.18 However, treatment with WP1130 resulted in an increased association between Bcr-Abl and Hsp90 (Figure 2C), suggesting a distinct mechanism for Bcr-Abl down-regulation. We further assessed the impact of WP1130 and 17-AAG on cellular ubiquitinated protein and Hsp70 protein levels, because Hsp90 inhibition results in...
Hsp70 induction. 17-AAG had no effect on ubiquitinated protein levels, but led to the induction of Hsp70 (Figure 2D). WP1130 induced a rapid increase in ubiquitinated proteins in the detergent-soluble cell fraction, with substantial accumulation in the detergent-insoluble fraction at later time points. Hsp70 levels were also increased in WP1130-treated cells, but based on the enhanced association of Hsp90 with Bcr-Abl in WP1130-treated cells, Hsp90 does not appear to be inhibited, and therefore is not directly associated with Hsp70 induction (Figure 2C).

Because ubiquitination can mediate protein degradation and intracellular trafficking, we examined Bcr-Abl content in

Figure 3. WP1130 induces cellular trafficking of Bcr-Abl and inhibits its substrate phosphorylation in CML cells. (A) BV-173 cells were treated with 5 μM WP1130 for the intervals indicated before assessing Bcr-Abl and other protein levels in total cell lysates and in the detergent-soluble and -insoluble fractions. Actin was blotted as a protein-loading control. (B) BV-173 and BV-173R cells were treated with 5 μM WP1130 for 2 hours before detergent-soluble and -insoluble cell lysates were blotted for Bcr-Abl (K12 antibody) and actin as a protein-loading control. (C) KS62R cells were treated with 5 μM WP1130 for 2 hours before proteins derived from the total cell lysate (T) and detergent-soluble (S) and insoluble (I) lysates were probed for Bcr-Abl and other proteins or phosphoproteins as indicated. Total cell lysates represent the detergent-soluble and -insoluble lysates combined. Actin served as a protein-loading control. (D) BaF3 cells transformed with eGFP-Bcr-Abl with the T315I mutation were treated with 5 μM WP1130 for the intervals noted before detergent-soluble cell lysates were immunoblotted for the indicated protein or phosphoprotein. Actin was blotted as a protein-loading control. Loss of Bcr-Abl from the soluble (cytoplasmic) cell fraction was associated with reduced signaling (pY-Stat5) and the onset of apoptosis (cleaved PARP). (E) Mononuclear cells from 2 CML patients who were progressing on imatinib therapy were treated with 5 μM WP1130 for 4 hours before cell lysates representing the combined detergent-soluble and -insoluble lysates were blotted for Bcr-Abl by immunoblotting (top). The detergent-soluble cell lysate was also immunoblotted for Bcr-Abl substrate phosphoproteins (pY-Stat5, pY-CrkL), as well as Stat5 and CrkL total protein levels. (F) CD34+ cells from a healthy donor and 2 imatinib-refractory CML patients were treated with the indicated concentrations of WP1130 for 24 hours before analysis of cell survival by annexin/propidium iodide staining and flow cytometry. The results represent the average ± SD of 3 replicates. Statistical significance was determined with a paired Student t test.

Hsp70 induction. 17-AAG had no effect on ubiquitinated protein levels, but led to the induction of Hsp70 (Figure 2D). WP1130 induced a rapid increase in ubiquitinated proteins in the detergent-soluble cell fraction, with substantial accumulation in the detergent-insoluble fraction at later time points. Hsp70 levels were also increased in WP1130-treated cells, but based on the enhanced association of Hsp90 with Bcr-Abl in WP1130-treated cells, Hsp90 does not appear to be inhibited, and therefore is not directly associated with Hsp70 induction (Figure 2C).

Because ubiquitination can mediate protein degradation and intracellular trafficking, we examined Bcr-Abl content in
WP1130-treated cells. As shown in Figure 3A, WP1130 treatment resulted in a rapid and near complete trafficking of Bcr-Abl from the detergent-soluble to the detergent-insoluble cell fraction. Compartmentalization of Bcr-Abl into the insoluble fraction was associated with loss of phosphorylation of Stat5 without affecting Jak2 (Figure 3A). However, no significant change in the Bcr-Abl protein content in whole-cell extracts was noted. Increased Bcr-Abl protein content in the detergent-insoluble cell fraction was also observed in imatinib-resistant CML cells, such as BV-173R expressing T315I-Bcr-Abl (Figure 3B) and K562R overexpressing Lyn kinase7 (Figure 3C). Neither Jak2 nor Lyn detergent solubility was affected by WP1130 in these cells.

In BaF3 transfectants expressing Bcr-Abl, loss of Bcr-Abl from the detergent-soluble fraction was associated with reduced substrate phosphorylation (pY-Stat5) and the onset of apoptosis (PARP cleavage; Figure 3D). Bcr-Abl in primary CML cells from imatinib-resistant patients was also observed to translocate to the detergent-insoluble fraction after WP1130 incubation, and was associated with a loss of Bcr-Abl substrate (pY-Stat5, pY-CrkL) phosphorylation (Figure 3E). The detergent-insoluble fraction is highly enriched in cytoskeletal proteins and components of cellular structures called aggresomes (data not shown).32 These results suggest that WP1130 blocks Bcr-Abl substrate phosphorylation through compartmentalization of Bcr-Abl.

To determine whether WP1130 induces differential effects on protein ubiquitination and survival of CD34+/H11001 cells from both healthy donors and CML patients, primary CD34+/H11001 cells were isolated and treated with WP1130 for 4 hours before total cell lysates were subjected to immunoblotting for ubiquitin. High-molecular-weight ubiquitinated proteins were increased by WP1130 in both normal and leukemic CD34+ cells (Figure 3F). However, CD34+ cells from CML patients were significantly more sensitive to WP1130-mediated apoptosis than cells from healthy donors (Figure 3G). This may be related to the affects of WP1130 on Bcr-Abl compartmentalization and signal transduction. However, because only a 2-fold difference in half-maximal inhibitory concentration (IC50) values was noted between CML (~2.8μM) and normal donors, further studies are needed to determine whether WP1130 blocks Bcr-Abl substrate phosphorylation through compartmentalization of Bcr-Abl.
and normal CD34+ cells (>5 μM), it is unclear whether this compound is a candidate for clinical studies. WP1130 derivatives with greater water solubility and a more restricted DUB-inhibitory profile are being designed and tested. We have also noted that the Bcr-Abl compartmentalization and apoptotic activity of WP1130 are engaged within 1 hour of exposure and cannot be reversed by removal of the compound (supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). These results suggest that the effects of WP1130 are irreversible and that cells will require only minimal contact time to elicit a response.

Bcr-Abl is ubiquitinated in WP1130-treated CML cells

Ubiquitinated protein content can be increased as a consequence of proteasome inhibition, loss of protein chaperone activity, or increased cellular oxidation leading to protein misfolding. However, we did not detect the loss of Hsp90 association with Bcr-Abl (Figure 2C and Figure 4A) and did not find elevated ROS in WP1130-treated cells (Figure 4B). Further, WP1130 did not suppress either 20S proteasome activity in intact CML cells or purified 20S proteasome subunit preparations, eliminating the possibility that WP1130 acts as a proteasome inhibitor (Figure 4C). WP1130 was compared with the potent 20S proteasome inhibitor bortezomib to determine whether they both had an impact on protein ubiquitination and Bcr-Abl compartmentalization. Both compounds increased ubiquitinated protein content, but only WP1130 induced accumulation of detergent-insoluble ubiquitinated proteins (Figure 4D). WP1130 treatment led to the reduction of Bcr-Abl from the detergent-soluble fraction and the appearance of Bcr-Abl in the detergent-insoluble fraction, but no change in total Bcr-Abl content was noted. Bortezomib elevated ubiquitinated protein levels but had no affect on Bcr-Abl detergent solubility or protein levels.

To determine whether Bcr-Abl was ubiquitinated in WP1130-treated cells, we treated CML cells with WP1130 for short intervals (30 minutes) to allow recovery of Bcr-Abl from the detergent-soluble fraction (for direct immunoprecipitation). Bcr-Abl immunoblotting after immunoprecipitation from WP1130-treated cells demonstrated a moderate reduction in Bcr-Abl recovery from the detergent-soluble extract (Figure 5A left), but a marked increase in its ubiquitination. Blotting confirmed a significant increase of Bcr-Abl and ubiquitinated proteins in the insoluble fraction from WP1130-treated cells (Figure 5A right). To determine whether Bcr-Abl modification by WP1130 was due to the transfer of specific ubiquitin polymers to the kinase, soluble cell lysates were subjected to affinity enrichment for K48-linked (ataxin) or K63-linked (Rap80) ubiquitin polymers, and protein eluates were immunoblotted for Bcr-Abl. Recovery of Bcr-Abl/K63-linked ubiquitin polymers was more prominent in WP1130-treated cells (Figure 5B), suggesting that increased K63-linked ubiquitin polymers on Bcr-Abl underlies the signal for its translocation into detergent-insoluble complexes.
Figure 6. WP1130 affects cellular DUB activity.

(A) Lysates (5 µg) from untreated (control) or WP1130-treated (5 µM, 4 hours) cells were incubated with 1 µg of K48-linked (left) or K63-linked (right) free chains of polyubiquitin (Ub1-5) for 10 minutes at 37°C. The extent of free chain hydrolysis by active DUB in each lysate was examined by ubiquitin immunoblotting. Ubiquitin polymer standards (Ub1-5) were resolved on the left and actin was immunoblotted as a protein-loading control. (B) K562 cells treated with 5 µM WP1130 (0 to 8 hours) were lysed in DUB-labeling buffer as described in “Ub-AMC protease assay.” Clarified supernatant (20 µg) was incubated with 200nM HA-UbVs (Boston Biochem) for 1 hour at 37°C. HA immunoblotting was used to assess changes in DUB activity (top). Tubulin was probed as a protein-loading control (bottom). The level of the upper HA-labeled band (arrow) was reduced by WP1130 and was determined to represent Usp9x (molecular weight, 293 kDa). The blot was reprobed for Usp9x as a measure of its protein-loading level (middle blot). (C) Usp9x was immunoprecipitated from K562 cell lysates, washed, and incubated in DUB assay buffer containing NEM (5mM), WP1130 (5 µM), or DMSO in a 100-µL reaction volume for 30 minutes at 37°C in 96-well fluorometry plates. K562 cell lysates immunoprecipitated with rabbit IgG were used as a control. After incubation, 500nM Ub-AMC was added to the reaction and the release of AMC-fluorescence was recorded over time. The representative fluorescence change over time is shown. The Usp9x activity results represent the average ± SD of triplicate assays. Similar results were obtained in 2 additional independent experiments. (D) K562 and K562R cells were treated with WP1130 for the intervals noted before total cell lysates were subjected to Mcl-1 and actin immunoblotting. (E) In vivo K562 cells were left untreated or treated with 5 µM imatinib, 0.5 µM dasatinib, 0.5 µM TG101209, or 5 µM WP1130 for 4 hours before cell lysates were analyzed for Usp9x activity by incubation with HA-UbVs and HA immunoblotting (as described in panel B). In vitro K562 cell lysates were treated with DMSO (control) or 5 µM WP1130 for 30 minutes at 37°C before assessing Usp9x activity by HA-UbVs labeling followed by HA blotting. The top portion of the HA immunoblot containing Usp9x is shown. The membrane was stripped and immunoblotted for Usp9x and actin. (F) DWD-2 cells were treated with WP1130 alone or pretreated with imatinib (5 µM), dasatinib (0.5 µM), or TG101209 (0.5 µM) for 1 hour before additional incubation in the presence of WP1130. All cells were harvested 4 hours after WP1130 treatment and total cell lysates were subjected to Mcl-1 immunoblotting. Actin was immunoblotted as a protein-loading control. Left, Control and shRNA-expressing BV-173 cells (as indicated) were treated with the indicated concentration of imatinib for 72 hours before assessing viability by MTT staining. The results represent the average ± SD of 4 replicates. Statistical significance was determined with a paired Student’s t test. *P < .05 for shRNA-Usp9x compared with Control or shRNA-Con. Right, BV-173 cells (control and shRNA-expressing as noted) were treated with the indicated concentration of ABT-263 for 72 hours before assessing viability as described in the center panel. The results represent the average ± SD of 4 replicates. Statistical significance was determined with a paired Student’s t test. *P < .05 for shRNA-Usp9x compared with Control or shRNA-Con.
To further assess Bcr-Abl ubiquitination and cellular distribution, eGFP-Bcr-Abl BaF3 transformants were examined by confocal microscopy. Treatment with WP1130 resulted in the clustering of Bcr-Abl and ubiquitin in juxtanuclear complexes (white arrows) resembling aggresomes. Biochemical studies support the inclusion of other proteins defining the aggresome in complex with Bcr-Abl from WP1130-treated cells (data not shown). These results suggest that WP1130 stimulates ubiquitination of Bcr-Abl (primarily with K63-linked polymers), leading to its transfer to the aggresome.

WP1130 inhibits DUB activity

A cross-conjugated αβ-unsaturated dienone with 2 sterically accessible electrophilic β-carbons is a molecular determinant of isopeptidase or DUB-inhibitor activity. The presence of these determinants in WP1130 prompted an investigation of its potential DUB-inhibitor activity. Lysates derived from untreated or WP1130-treated cells were incubated with purified ubiquitin polymers (Ub1-Ub5), and the relative recovery of polymers was assessed by immunoblotting. Lysates derived from WP1130-treated CML cells showed a partial protection of both K48- and K63-linked ubiquitin polymers from disassembly, supporting the possibility of DUB inhibition by WP1130 (Figure 6A). To more closely examine DUB inhibition, lysates from untreated, WP1130-treated (1-8 hours), or NEM-treated CML cells were incubated with fluorescent DUB substrate (Ub-AMC) and activity was monitored over time. Lysates derived from cells treated with the nonspecific DUB inhibitor NEM completely blocked substrate hydrolysis (supplemental Figure 2A), whereas lysates from WP1130-treated CML cells showed limited effects on Ub-AMC hydrolyzing activity. These results suggested that WP1130-mediated ubiquitin changes may be mediated through inhibition of a limited subset of DUBs.

To examine the impact of WP1130 on specific DUB activity, CML cells were treated with WP1130 and lysates were incubated with the HA-tagged, irreversible DUB substrate HA-UbVs, as described previously. The covalent modification of DUBs by this construct allows analysis of DUB activity by HA immunoblotting. Inhibition of DUB activity by WP1130 may be detected by a reduction in HA labeling of WP1130-sensitive DUBs. As shown in Figure 6B, lysates from WP1130-treated cells showed a reduced capacity to label one prominent high–molecular weight DUB identified as Usp9x (Figure 6B arrow). Usp9x is predominantly expressed in the cytoplasmic, detergent-soluble fraction, and WP1130 treatment did not affect Usp9x recovery or protein content (Figure 6C bottom). To determine whether WP1130 directly affects Usp9x activity, K562 cell lysates were incubated with WP1130 for 1 hour before incubation with HA-UbVs DUB substrate and analysis by HA blotting. Usp9x labeling was completely blocked in the presence of WP1130, suggesting direct Usp9x inhibition.
Other DUBs were also affected by WP1130, suggesting that WP1130 targets additional DUBs with as yet unknown specificity or selectivity. To confirm direct DUB inhibition, Usp9x was immunoprecipitated, washed extensively, and incubated with WP1130 before assessing DUB activity using the fluorescent DUB substrate Ub-AMC (supplemental Figure 2A). The rate of substrate hydrolysis was measured and compared in DMSO and WP1130 incubation assays to estimate the percentage of DUB inhibition by WP1130. Normal IgG immunoprecipitates did not result in substrate hydrolysis compared with control reactions. WP1130 reduced Usp9x activity by > 80% (Figure 6C), demonstrating that WP1130 functions as a subset-specific DUB inhibitor with activity against Usp9x. Other DUB activities were completely insensitive to WP1130 inhibition when assessed by HA-UbVs labeling (supplemental Figure 3A) or in DUB enzyme assays with Ub-AMC substrate (supplemental Figure 3B).

Recent studies have demonstrated that Usp9x controls Mcl-1 deubiquitination and degradation. To determine whether Usp9x inhibition by WP1130 affects Mcl-1 levels in CML cells, total cell extracts were immunoblotted for Mcl-1. WP1130 reduced Mcl-1 levels (Figure 6D) in a temporal fashion that paralleled Usp9x inhibition, and was active in both imatinib-sensitive (K562) and imatinib-resistant (K562R) CML cells. To determine whether Bcr-Abl or Jak2 kinase inhibition could also reduce Usp9x activity, CML cells were treated with WP1130, a Bcr-Abl–selective inhibitor (imatinib), a Jak2-selective inhibitor (TG101209), or a multi-kinase inhibitor (dasatinib) for 4 hours before cell lysates were subjected to HA-UbVs labeling and direct Usp9x immunoblotting. In vitro treatment of cell lysates with WP1130 (5 μM, 30 minutes) was also included to confirm direct Usp9x inhibition (Figure 6E last 2 lanes). WP1130 effectively reduced Usp9x activity, whereas none of the kinase inhibitors examined had significant Usp9x-inhibitory activity. The effect of kinase inhibition on Mcl-1 protein levels was also examined in WTD-2 CML cells and compared with the effects of WP1130 alone or in combination with kinase inhibitors. As shown in Figure 6F, WP1130 treatment resulted in a rapid reduction in Mcl-1 protein levels and was associated with Usp9x inhibition (supplemental Figure 3A). Bcr-Abl kinase inhibitors (imatinib and dasatinib) also reduced Mcl-1 levels, but to a lesser extent than that detected in WP1130-treated cells. Interestingly, the Jak2 inhibitor TG101209 did not reduce Mcl-1 protein levels, suggesting that Bcr-Abl, but not Jak2, plays a role in the control of Mcl-1 expression in CML cells. WP1130 caused a greater reduction in Mcl-1 levels than kinase inhibitors alone, suggesting that the combined effects of WP1130 on Bcr-Abl signaling and Usp9x activity in CML cells induces greater Mcl-1 down-regulatory activity than that achieved through Bcr-Abl kinase inhibition alone.

BV-173 cells expressing shRNA directed against Usp9x were used to determine the effects of this DUB on apoptosis initiated by Bcr-Abl kinase inhibition (imatinib) or by a BH3 mimetic (ABT-263), as described previously. Usp9x-shRNA–expressing cells showed reduced levels of Mcl-1 and ~ 2-fold increased sensitivity to imatinib compared with controls (IC₅₀ ~ 300nM in shRNA-Usp9x–expressing cells vs ~ 600nM in shRNA controls; Figure 6G). Cells with reduced levels of Usp9x were also more sensitive to intermediate concentrations of ABT-263.

A summary of the effects of WP1130 on CML cells is shown in Figure 7. WP1130 induces Bcr-Abl ubiquitination and directly inhibits Usp9x activity. However, although WP1130 inhibits Usp9x activity, this does not appear to be responsible for the observed increase in Bcr-Abl ubiquitination. These 2 activities led to a reduction in Bcr-Abl signaling and induction of apoptosis, because aggresomal Bcr-Abl was unable to phosphorylate downstream proteins, whereas Usp9x inhibition reduced apoptotic thresholds through its control of Mcl-1 protein levels. This mechanism of control over tumor cell survival and signaling through changes in the activity of ubiquitin cycle–regulatory proteins has not been described previously and may be of significance in tumors that are dependent on continuous kinase activity and elevated Usp9x activity.

Discussion

In this study, we analyzed the mechanism of action of WP1130, a small molecule with previously reported Bcr-Abl down-regulatory activity. By screening multiple kinases expressed and activated in CML cells, we could detect only Bcr-Abl down-regulation in CML cells (Figure 1A). Thorough examination showed that WP1130 action is initiated in the cytoplasmic fraction of the cell (Figure 2D), which may partially explain the selectivity for the predominantly cytoplasmic Bcr-Abl protein. Other kinases, such as Jak2, Lyn, and PI3-K, are reported to be detected in the membrane fraction or associated with transmembrane receptors. Analysis of several tumor types supports the observed selectivity of WP1130 for specific cytoplasmic kinases, because transmembrane kinases such as the HER family, c-kit, and Fli-3 are insensitive to WP1130. However, in hematologic tumors that do not express Bcr-Abl, Jak2 undergoes rapid ubiquitination and aggresomal trafficking in response to WP1130. The reason for this change in kinase target in Bcr-Abl–negative cells is unknown, but may be due to differential expression and activity of DUB in Bcr-Abl–transformed cells (N.J.D., manuscript in preparation). Jak2 ubiquitination also explains the reported Jak/Stat-inhibitory activity of WP1130 (V.K., N.J.D., manuscript in preparation) and less-active derivatives such as WP1066 and WP1034. Based on the activities reported here and on previous observations, WP1130 initiates a series of events that results in the ubiquitination of proteins such as Bcr-Abl with K63-linked polymers. This form of protein modification typically signals for proteins to traffic or transfer to organelles, and, in the case of Bcr-Abl, the aggresome appears to be the main site for transfer. Aggresomes are typically formed in response to protein misfolding or overload and are proposed to be cytoprotective by reducing the potential for excess protein to interfere with cellular metabolism. However, in the case of Bcr-Abl, the aggresome appears to be the main site for transfer. In this regard, WP1130-initiated compartmentalization of Bcr-Abl converts a cytoprotective process into a cytotoxic one. Based on the observed inhibition of DUB activity by WP1130, it appears that DUB targets may be associated with the observed WP1130 activity. However, other potential targets must also be considered, and several approaches are being explored to define mediators underlying aggresome formation in WP1130-treated cells. We have also noted that WP1130, like imatinib, triggers CML cell autophagy, but this is not sufficient to fully cytoprotect CML cells from DUB inhibition–mediated apoptosis. However, as demonstrated with imatinib, inhibition of WP1130-induced autophagy increases CML cell sensitivity to WP1130-mediated apoptosis (data not shown), suggesting that cellular stress applied
through direct (imatinib) or indirect (WP1130) loss of Bcr-Abl signaling can engage the autophagic pathway to partially subvert CML cell death. Suppressing autophagy may be an effective strategy to increase CML cell sensitivity to both kinase and DUB inhibitor–mediated apoptosis.

The increase in ubiquitinated protein content in WP1130-treated cells appears to be mediated primarily through inhibition of DUB activity, but other activities may also be involved.\textsuperscript{35,46} We did note an increase in Hsp70 levels after WP1130 treatment, suggesting a potential role for Hsp90 inhibition in the cellular response. However, we did not detect the reduction in Hsp90:Bcr-Abl complexes (Figure 2C) that is reported to occur as a consequence of loss of Hsp90 chaperone activity. One possible explanation for this response is that misfolded proteins accumulate as a result of DUB inhibition and increased protein ubiquitination in WP1130-treated cells. The cell induces protein chaperones (such as Hsp70) in an attempt to restrict the formation of toxic protein aggregates.\textsuperscript{45} The kinetics of Hsp70 induction (Figure 2) and the onset of aggresome formation (Figure 5) support this possibility.

We did not observe a change in the activity of the Bcr-Abl–associated E3 ligase c-Cbl after WP1130 treatment, and its involvement in regulating Bcr-Abl ubiquitination appears remote, because we detected a predominant linkage with K63-linked ubiquitin polymers on Bcr-Abl that are not considered substrates for c-Cbl in ubiquitin ligase reactions.\textsuperscript{47} Bcr-Abl trafficking, not its proteasomal degradation, appears to underlie the major impact of WP1130 on CML cells and is aligned with previously described activities associated with increased K63-specific ubiquitin polymers on target proteins.\textsuperscript{31} Because Ubc13 (E2) is the only known enzyme involved in forming K63-linked polyubiquitin chains,\textsuperscript{38} this restricts the number of potential upstream components that provide K63-linked ubiquitin polymers for subsequent transfer to Bcr-Abl. Additional studies are being performed to determine the role of Ubc13 and other ubiquitin-conjugating or -activating enzymes in WP1130 activity against Bcr-Abl.

We have identified a subset of DUB enzymes that may underlie some of the protein ubiquitination and apoptotic activity of WP1130. Although we did not detect a major change in total DUB activity in CML cells after WP1130 administration (Figure 6B and supplemental Figure 2), we were able to detect inhibition of specific DUBs such as Usp9x. Usp9x activity was sensitive to WP1130 in assays from intact cells, cell lysates, and enzyme preparations at concentrations necessary to induce Bcr-Abl trafficking and apoptosis. Recent reports have suggested that Usp9x increases Mcl-1 stability, extending its half-life by preventing its proteasomal destruction through de-ubiquitination.\textsuperscript{21} This activity and the up-regulation of Usp9x are associated with poor prognosis in multiple myeloma patients, and may underlie chemoresistance in several additional cancers.\textsuperscript{21} We were able to demonstrate a reduction in Mcl-1 levels in WP1130-treated cells that paralleled inhibition of Usp9x activity (Figure 6). Mcl-1 down-regulation combined with loss of Bcr-Abl signaling activity may be key contributors to the induction of apoptosis in WP1130-treated CML cells.

To determine the impact of Usp9x inhibition on CML cell survival, we silenced Usp9x in CML cells and demonstrated Mcl-1 down-regulation, which increased sensitivity to imatinib and other apoptotic stimuli (Figure 6G), but no affect on Bcr-Abl ubiquitination or its cellular localization was observed (data not shown). Other reports have suggested an association between Bcr-Abl and cytoskeleton-mediated Stat activation.\textsuperscript{24} In the present study, kinase inhibitors did not block Usp9x activity in CML cells (Figure 6G), but did affect Mcl-1 protein levels, possibly through modulation of Mcl-1 gene expression. Until recently, the role of Usp9x as a modulator of Mcl-1 activity in transformed cells was unknown, but inhibitors of Usp9x may be of therapeutic importance in several settings. WP1130 may be highly suited for CML–based therapy because it has indirect effects on Bcr-Abl signaling through kinase sequestration into aggresomes and direct inhibition of Usp9x activity, which may be essential in stabilizing CML stem cell survival. In light of the importance of Usp9x in the control of Mcl-1 levels, compounds such as WP1130 may be useful in overcoming the apoptotic resistance associated with Usp9x activity and Mcl-1 protection.

Inhibition of other DUBs may also play a role in WP1130 activity. We have noted a broader spectrum of DUB inhibition by WP1130 in other tumor cell types (data not shown). It is possible that simultaneous inhibition of several DUBs may be necessary to induce changes in Bcr-Abl ubiquitination and trafficking. Other reagents that measure DUB activity may yield a more comprehensive assessment of DUB activity and sensitivity to WP1130 in various tumors.\textsuperscript{49,50} In addition, an unbiased determination of direct targets of WP1130 may be assessable through other techniques and will be important in understanding its activity.\textsuperscript{51} In either case, our studies with WP1130 identified a novel pathway to kinase inhibition and apoptosis in CML cells. Additional studies of the key components in this pathway and the role of DUB inhibition in this process may highlight important targets for future cancer therapy.

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Authorship

Contribution: H.S. and V.K. performed the majority of the research, analyzed data, and wrote parts of the manuscript; L.F.P. and D.F. performed specific assays and analyzed data; W.G.B. and G.B. performed research; M.T. provided clinical samples and analyzed data; and N.J.D. directed the scope of the study, analyzed data, and wrote the manuscript.

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