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Inhibition of Deubiquitinase Activity and Ubiquitination of Jak2 Blocks Cytokine Signaling and Induces Tumor Cell Apoptosis

Vaibhav Kapuria

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**Inhibition of Deubiquitinase Activity and Ubiquitination of Jak2
Blocks Cytokine Signaling and Induces Tumor Cell Apoptosis**

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**A
DISSERTATION**

**Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY**

By

Vaibhav Kapuria

May 2010

DEDICATION

This dissertation is dedicated to the ones who are closest to my heart: my family, whose encouragement, support and patience made this research fruitful.

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I would like to thank my advisor, Dr. Nicholas Donato, for his excellent guidance and support throughout my research period. I would also thank my supervisory committee members: Warren S. Liao; Ph.D, Stephanie S. Watowich; Ph.D, Richard J. Ford; M.D, Ph.D and Bryant G. Darnay; Ph.D, for their guidance, encouragement and critical analysis of my research. Additionally, I would like to thank Moshe Talpaz; M.D, for his continued support towards my research.

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ABSTRACT

The Jak-stat pathway is critical for cellular proliferation and is commonly found to be deregulated in many solid tumors as well as hematological malignancies. Such findings have spurred the development of novel therapeutic agents that specifically inhibit Jak2 kinase, thereby suppressing tumor cell growth. Tyrphostin AG490, the first described Jak2 inhibitor, displays poor pharmacology and requires high concentrations for anti-tumor activities. Our research group screened a small library of AG490 structural analogues and identified WP1130 as a potent inhibitor of Jak2 signaling. However, unlike AG490, WP1130 did not directly inhibit Jak2 kinase activity. Our results show that WP1130 induces rapid ubiquitination and subsequent re-localization of Jak2 into signaling incompetent aggresomes. In addition to Jak2, WP1130 also induces accumulation of other ubiquitinated proteins without inhibiting 20S proteasome activity. Further analysis of the mechanism of action of WP1130 revealed that WP1130 acts as a partly selective DUB inhibitor. It specifically inhibits the deubiquitinase activity of USP9x, USP5, USP14 and UCH37. WP1130 mediated inhibition of tumor-associated DUBs resulted in down-regulation of anti-apoptotic and up-regulation of pro-apoptotic proteins, such as MCL-1 and p53 respectively. Our results demonstrate that chemical modification of a previously described Jak2 inhibitor results in the unexpected discovery of a novel compound which acts as a DUB inhibitor, suppressing Jak-Stat signaling by a novel mechanism.

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A. Introduction

Janus kinase 2 (Jak2) is an oncogenic tyrosine kinase that plays a critical role in cytokine signal transduction and tumor cell survival. Deregulation of Jak2-dependent downstream signaling is common in many forms of cancer. Owing to its crucial role in supporting tumor proliferation, Jak2 represents an attractive candidate for targeted therapeutics in cancer. This dissertation is focused on studies of a small molecule, WP1130, which suppresses Jak2 signaling by a novel mechanism. WP1130 represents a second-generation derivative of AG490, a previously described inhibitor of Jak2 kinase. Although WP1130 resembles AG490 structurally, the inhibition of Jak2 signaling by WP1130 is distinct, involving ubiquitination of Jak2. Thorough investigation to gain molecular insights into the mechanism of action of WP1130 revealed that WP1130 acts as a deubiquitinase inhibitor, affecting cellular protein ubiquitination, including Jak2. To provide significance to the observations of WP1130's unique activity, the introductory section includes background on both Jak-Stat signaling and the protein-ubiquitination pathway.

A1. Sub-Section: Jak-Stat Signaling

(A1-1) Structure of Jak family proteins

Jaks are a family of non-receptor tyrosine kinases containing Jak1, Jak2, Tyk2 and Jak3 as members. While Jak1, Jak2 and Tyk2 are ubiquitously expressed in all tissues, Jak3 is expressed only in hematopoietic cells (1, 2). Structurally, the Jak family proteins are characterized by the presence of following domains: an N-terminal FERM (four point one, ezrin, radixin, and moesin), central SH2 (src-homology 2), pseudokinase and a C-terminal kinase (Illustration 1). The FERM domain of Jak

proteins is responsible for their interaction with cytokine/growth-factor receptors (3). Jak FERM domain also acts as a chaperone for some cytokine receptors such as EpoR, PrlR and oncostatin MR (4), thereby regulating the expression of these receptors. Jaks are also characterized by the presence of an N-terminal endogenous SH2 domain. Although SH2/SH3 domains are known to play a critical role in mediating protein-protein interactions, the role of the SH2 domain in Jak proteins is not yet fully characterized (5). A catalytically-active kinase domain is located at the c-terminus of the protein, preceding the enzymatically-inactive pseudokinase domain. Although the pseudokinase domain lacks any kinase activity, it regulates the overall kinase activity of the Jak proteins (6, 7). Recently, many activating mutations within the pseudokinase domain of Jak2 have been described which are associated with myeloproliferative disorders (See section 1.1D).

Owing to their association with cytokine receptors, Jaks are most commonly localized to cytokine receptors, as well as the plasma membrane. However, there have been reports describing nuclear localization of Jaks (8-10). Nuclear Jak2 has been shown to be kinetically active, where it plays a role in stabilization of nuclear factors (8) and histone phosphorylation (10).

The relevance of signal transduction events mediated by Jak family proteins are highlighted in the knockout (KO) phenotype of mice. While Jak1 knockout mice show defective lymphoid and neural development(11), the importance of Jak2 in hematopoiesis can be illustrated by defective erythropoiesis in Jak2-deficient mice, leading to embryonic lethality (12, 13). The Jak3 knockout mice revealed an exclusive role for Jak3 in maintaining host immunity. A severe reduction in T and B lymphocytes and Natural Killer cells is observed in Jak3 KO mice, leading to severe combined immune-

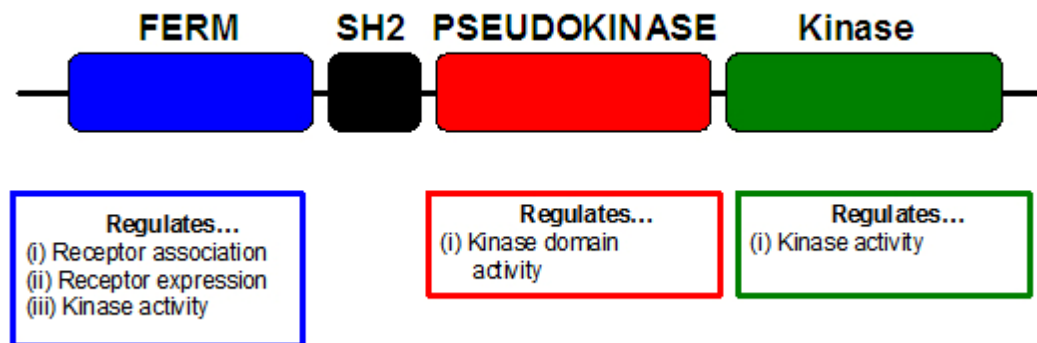


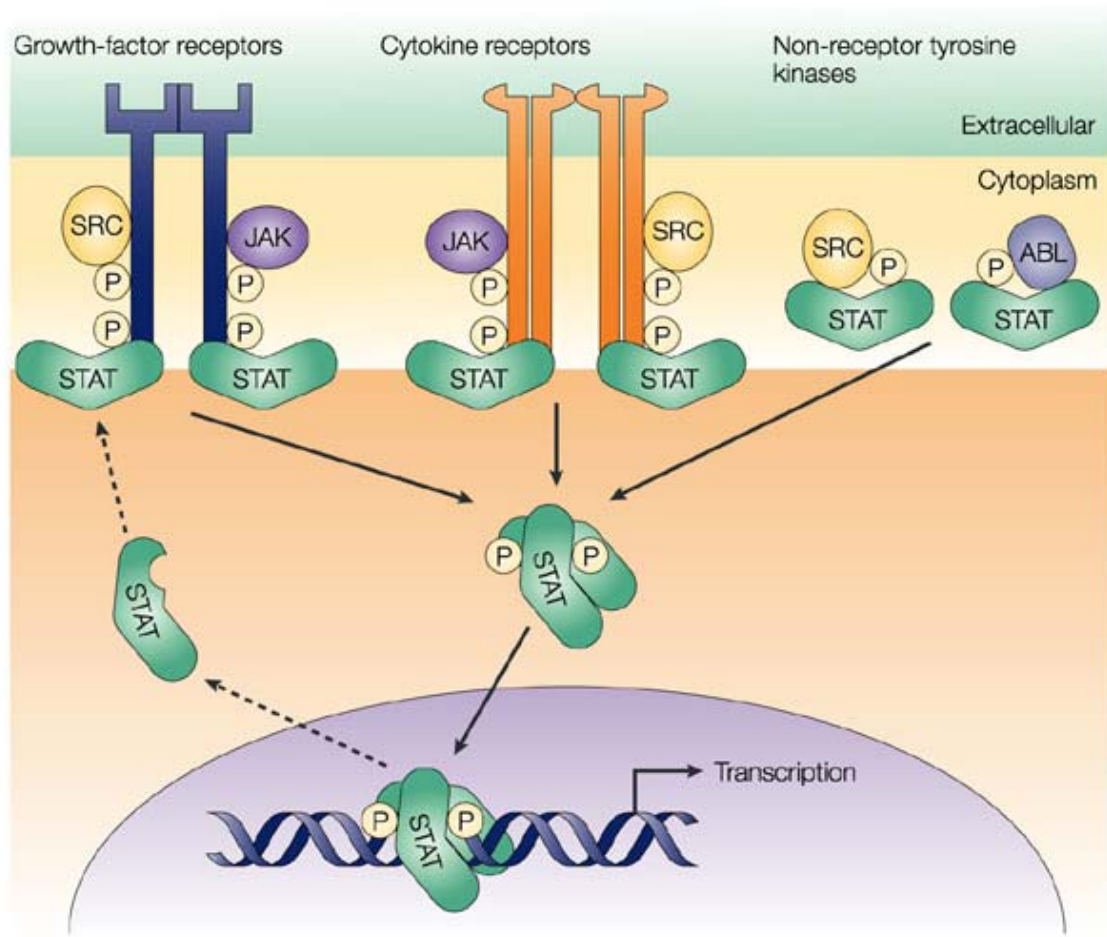
Illustration 1: Domains of Jak proteins and their role in signal transduction.

deficiency (14). Although Tyk2 KO mice are phenotypically normal, they show modest susceptibility to viral infections (15).

(A1-2) Jak-Stat Signal Transduction

Jak2 tyrosine kinase plays a critical role in cytokine signaling in hematopoietic cells induced by growth factors such as growth hormone (GH), prolactin (PRL), erythropoietin (EPO) and cytokines including IL-3/-5/-6 and interferon (IFN) (16, 17). Binding of the cytokines to their cognate receptor leads to receptor homo/hetero-dimerization, leading to juxtaposition of receptor associated Jak2. This allows to trans-phosphorylation of Jak2 within its activation loop [Tyrosine 1007/1008 for Jak2 (18)], thereby increasing their catalytic activity. Activated Jak2 in turn phosphorylates critical tyrosines on the receptor, creating docking site for latent cytoplasmic proteins which relay the downstream signal. One such family of transcription factors is the signal transducers and activators of transcription proteins (Stat). Jak2 phosphorylates receptor-bound Stats, leading to their dimerization and subsequent translocation to the nucleus, where they up regulate the transcription of several genes involved in cellular growth, cell cycle as well as anti-apoptotic mechanisms (Illustration 2) [for reviews see (19-21)]. Although Stat proteins are key mediators of Jak signalling, they can also be activated by other tyrosine kinases such as non-receptor tyrosine kinases including src (22), Bcr-Abl (23) or receptor tyrosine kinases such as EGFR (24) and PDGFR (25).

The Stat family comprises of seven related proteins – Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 (26). Stats play a dual role in signaling events by acting as secondary messengers as well as nuclear factors to relay message from the upstream kinase. As nuclear factors, Stats regulate a diverse group of genes, *Bcl-xL*, *cyclin D1*, *p21* and *c-myc* (27-29), which are known to express anti-apoptotic/pro-survival proteins.



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Illustration 2: Jak-Stat Signaling. Binding of the cytokine/growth factor to its cognate receptor leads to the activation of Jak kinases, which in turn phosphorylate Stat, leading to their dimerization. Stats can also be activated independent of Jak, by cytosolic kinases such as Abl and Src. Dimers of Stat then translocate to the nucleus, regulating the transcription of downstream target genes which play a crucial role in cell growth and survival.

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(A1-3) Negative-Regulation of the Jak-Stat Pathway

Jak-Stat signaling is subject to multiple levels of regulation to prevent excessive signalling and abnormal cellular activation that could lead to malignant transformation. Stat signaling can be attenuated by several mechanisms including cytokine receptor internalization, activation of tyrosine phosphatases, binding of the protein inhibitors of activated Stats (PIAS) to Stats and the expression of members of the suppressors of cytokine signaling (SOCS) family (Illustration 3).

Tyrosine Phosphatases:

As Jak-Stat signaling is mainly activated via a series of tyrosine phosphorylation events, protein tyrosine phosphatases (PTP) play a crucial role in regulating the magnitude and duration of the signal. PTPs with reported involvement include SH2-domain containing tyrosine phosphatases such as SHP1, SHP2; CD45 and PTP1B. While SHP1/2 and PTP1B are cytosolic, CD45 is a transmembrane phosphatase. SHP1 binds to phosphorylated Jak and receptor chains, subsequently dephosphorylating the substrate. SHP1 KO mice display hypersensitivity to cytokines such as GM-CSF and IL-3 (30). Mice deficient in CD45 show hyperactivation of the Jak-Stat pathway and robust responses to stimulation from cytokines such as IL-3, IL-4 and EPO (31). PTP1B directly binds and dephosphorylates Jak2 and PTP1B KO mice display hypersensitivity to leptin activation (32). De-regulations of PTP mediated negative feed-back, such as silencing of the *shp1* gene by promoter methylation have been reported in various kinds of leukemia, lymphomas (33) and myeloma (34).

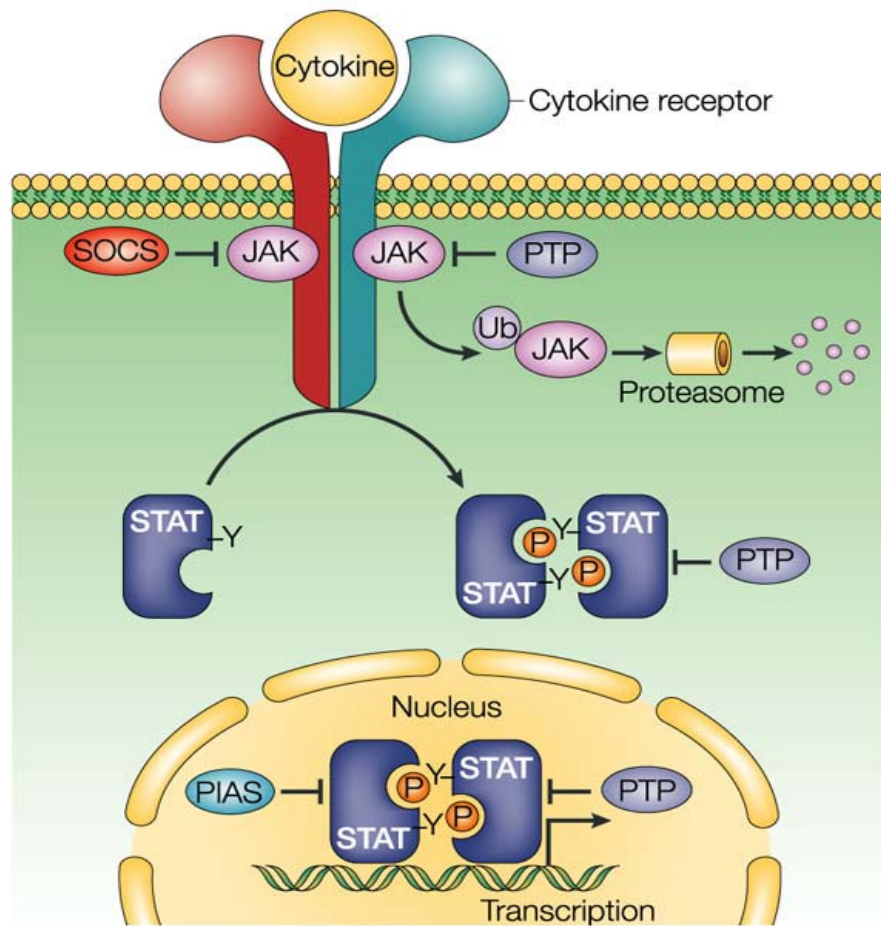
Protein Inhibitors of Activated Stats:

PIAS are negative regulators of Stat signaling, possessing a small ubiquitin-like modifier (SUMO)-E3-ligase activity. They block the transcriptional activity of the Stats, without affecting their phosphorylation, by blocking their DNA-binding activity (35) or by

recruiting co-repressor proteins such as histone deacetylases (HDAC) (36). Additionally, PIAS also promote Stat sumoylation which may inhibit their signaling (37).

Suppressors of Cytokine Signaling:

SOCS proteins are cytokine-inducible negative regulators of Jak-Stat signaling. They are distinct from PIAS and PTPs which are constitutively expressed in the cell. SOCS proteins inhibit Jak–Stat signalling by competing with Stats for the binding sites on the receptor. SOCS also directly bind to Jak and inhibit their activity, which can be attributed to the presence of a kinase inhibitory region within their structure. Lastly, SOCS suppress Jak-Stat signaling by promoting the ubiquitination and subsequent degradation of Jak proteins (38). SOCS1 interacts with a variety of proteins involved in the ubiquitin pathway to promote the degradation of Jak [Reviewed in (39)]. SOCS proteins also act as tumor-suppressors (40, 41) and the loss of SOCS expression by promoter methylation (42) has been associated with tumorigenesis. Recent reports also described hyperphosphorylation of SOCS3 in myeloproliferative disorders by mutant Jak2 (V617F), compromising its activity to regulate the constitutively active Jak2 pathway (43).



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Illustration 3: Negative Regulation of Jak-Stat Pathway. Jaks are regulated by both PTPs and SOCS proteins. PTPs dephosphorylate activated Jaks; while SOCS promote proteasomal degradation of Jaks. Activated Stats are regulated at the level of PTPs and PIAS.

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(A1-4) Deregulation of the Jak-Stat pathway in Cancer

Given the essential role for Jak-Stat signaling in the growth-promoting activities of a number of cytokines, constitutive activation of Jaks would be expected to have pathological consequences. The Jak-Stat pathway is often found to be activated in most forms of cancer [Reviewed in (44), (45)]. Constitutive activation of Jak2 kinase, either through cytokine-autocrine signaling (46) or via formation of Jak2 fusion proteins has been reported to play a role in oncogenesis (47, 48). Additionally, activating mutations in Jak2 pseudokinase/kinase domain have been recently implicated in myeloproliferative disorders (49, 50).

Typically, in oncogenesis associated with Jak2 chromosomal translocation, the kinase domain of Jak2 is found to be fused to an amino terminal domain derived from a fusion gene which contains a dimerization domain. The resulting fusion protein is constitutively active, promoting uncontrolled cell growth even in the absence of any cytokine or growth-factor. The first Jak2 chromosomal translocation to be identified involved a translocation between Jak2 kinase domain and helix-loop-helix domain of TEL (transcription factor). The resulting hyperactive fusion protein, TEL-Jak2 [t(9;12)(p24;p13)], was demonstrated to play a role in B-cell acute lymphoid leukemia and atypical chronic myeloid leukemia (CML) (47), (51). The TEL-Jak2 protein exhibits constitutive tyrosine kinase activity and its expression induces transformation of Ba/F3 cells through IL3-independence (47). Other fusion proteins of Jak2, such as Bcr-Jak2 [t(9;22)(p24;q11.2)] (52), PCM1-Jak2 [t(8;9)(p22;p240)] (48) have also been identified in cases of atypical (CML).

As described earlier in section A1-1, the pseudokinase domain of Jak2 is reported to serve a regulatory role. Therefore, mutations in the pseudokinase domain which suppress the auto-inhibitory function of the pseudokinase domain may result in

Table 1: Fusion proteins/mutations of Jak2 and their implication in cancer.

Mutation type	Mutation	Phenotype	Year identified
Translocation	TEL-Jak2	ALL, aCML	1997 ^(47, 48)
Translocation	PCM1-Jak2	aCML, AML, ALL	2005 ⁽⁵¹⁾
Translocation	BCR-Jak2	CML	2005 ⁽⁵⁰⁾
Substitution	Jak2-V617F	PV, ET, PMF	2005 ⁽⁵⁴⁻⁵⁷⁾
Substitution	Jak2-T875N	Megakaryoblastic leukemia	2006 ⁽⁵⁸⁾

ALL - Acute Lymphoblastic Leukemia; aCML - Atypical Chronic Myeloid Leukemia; AML - Acute Myeloid Leukemia; PV - Polycythemia Vera; ET - Essential Thrombocytopenia; PMF - Primary Myelofibrosis

constitutively active Jak2 kinase. The first report describing mutations within Jak2 which are associated with a pathological phenotype was reported using *Drosophila* Jak homolog, Hop (53). A point mutation in the pseudokinase domain of Hop was associated with a hyperactive Hop kinase and a hematopoietic malignant phenotype in flies (54). The relevance of Jak2 mutation in human diseases was further strengthened by studies which described short interfering RNA (siRNA)-mediated knock-down of Jak2, leading to impaired colony formation in patients with polycythemia vera (PV) (55). This observation was later confirmed by several independent groups which reported a correlation between a clonal mutation in the Jak2 pseudokinase domain (Jak2 V617F) and myeloproliferative disorders, such as essential thrombocytopenia, myelofibrosis and PV (49, 50, 56). Another novel mutation, Jak2T875N, was also identified in acute megakaryoblastic leukemia (57).

Collectively, the studies mentioned above indicate that the Jak2 chromosomal rearrangement and kinase activating point mutations are associated with hematological malignancies. These Jak2 gene aberrations in cancer are summarized in Table 1.

(A1-5) Small molecule inhibitors of Jak2-Stat3 Signaling

Many tumors are addicted to oncogenic proteins which play a crucial role in their survival. The “addiction” to these oncogenic proteins significantly increases the prospects of targeting them for therapeutic purposes. Malignancies such as CML harbor the Philadelphia chromosome which is present only in transformed cells and leads to the formation of oncogenic Bcr-Abl kinase (58). This observation led to the development of small molecules that could disrupt Bcr-Abl kinase activity; thereby sensitizing the tumors to apoptosis. However, the human genome codes for nearly 500 kinases, most of which possess conserved catalytic domains and ATP-binding pocket. Hence, the

generation of small molecule inhibitors which selectively target one or a small subset of these kinases without obvious off-target effects seemed challenging. However, the development of gleevec for the successful treatment of Bcr-Abl positive-CML (59) has provided the proof of principle needed to further develop small molecule inhibitors to other oncogenic kinases as well. The epidermal growth factor receptor (EGFR) is another receptor tyrosine kinase, which is highly expressed and mutated in cancers (60); thereby validating the development of gefitinib and erlotinib, for non-small cell lung cancer and pancreatic cancer (61, 62).

The discovery of gain-of-function Jak2 mutations (V617F) in PV and other hematological disorders spurred the interest of the scientific community to develop more potent Jak2 inhibitors. Since 2005, many small molecule compounds [TG101348, Z3 (63), CP-690,550 (64)] have been described to act as Jak2 inhibitors, conferring anti-tumor activities at low nM concentrations. TG101348 potently inhibits Jak2 tyrosine kinase, and suppresses the proliferation of human erythroleukemia cells, which express the Jak2-V617F mutation (65), with considerably less off-target effects against related kinases, such as Jak3. Treatment of mice expressing Jak2-V617F mutant kinase with TG101348 reduced the growth of hemopoietic colonies from primary progenitor cells. Currently, the TG101348 compound is undergoing successful phase I/II clinical trials in major cancer centers around the world (66). Additional Jak2 inhibitors such as CEP701 (67) and INCB18424 (68) are undergoing clinical trials.

Before the development of successful and high affinity Jak2 kinase inhibitors, driven by the discovery of kinase activating Jak2 mutation in myeloproliferative disorders, Tyrphostin AG490 was the first described inhibitor of Jak2. This compound was shown to induce apoptosis in acute lymphoblastic leukemia (69). Although AG490 displayed anti-tumor properties against many forms of cancer (70, 71), its clinical relevance was limited due to lack of specificity (72) and requirements of high μM

concentrations for effective Jak2 inhibition. To circumvent this problem, our research group screened a small library of AG490 structural analogues for their ability to suppress Jak2/Stat3 signaling using cell based assay. Further structure-activity relationship (SAR) based screening lead to the design and synthesis of a lead compound, WP1130, which could effectively suppress Jak2 driving Stat activation at high nM to low μ M concentrations. The anti-tumor effects of WP1130 and less active derivatives (WP1066, WP1034) against various tumors have already been reported in CML (73), melanoma (74), glioblastoma (75) and myeloproliferative disorders (76). An earlier report by Ferrajoli et al (77) described down-regulation of Jak2 by WP1066, which results in the loss of Stat3 activation and eventually induces apoptosis. Down-regulation of Jak2 kinase was reported as the mechanism of action of WP1066. However, no details of the Jak2 down-regulatory mechanism were provided.

A2. Sub-Section: Protein-Ubiquitination Pathway

(A2-1) Protein Ubiquitination System

Post-translational modifications of cellular proteins are induced by a vast variety of stimuli and upstream signaling events in cells. Although many different forms of post-translational modification of proteins have been identified to date, two post-translational modifications have emerged as key players in regulating signal transduction pathways. These include protein phosphorylation, which involves modification by a phosphate group, and protein ubiquitination, involving covalent attachment of ubiquitin to target protein. Most often, phosphorylation and ubiquitination precede each other, to regulate an individual protein and its cellular activities; suggesting a cross-talk between the two post-translational modifications.

Ubiquitin is a 76-amino acid protein (~ 8.5 kDa) which is constitutively and universally expressed in all eukaryotic cells. Prominent structural features of ubiquitin include its C-terminal glycine and the presence of 7 lysine residues. Ubiquitination refers to a covalent modification of cellular proteins in which an isopeptide bond links the C-terminal glycine of ubiquitin to lysine on the target proteins. The conjugation of ubiquitin to target protein involves a complex enzymatic system involving ubiquitin activating enzyme (E1), ubiquitin conjugases (E2) and E3 ubiquitin ligases. Although protein ubiquitination is known to regulate a myriad of cellular activities, the most prominent function of ubiquitin is the labeling proteins for their proteasomal degradation [Reviewed in (78)]. Similar to ubiquitin, other ubiquitin-like proteins (UBL) such as ISG15, FAT10, NEDD8 and SUMO follow an evolutionary similar evolutionary tract but regulate protein functions through distinct enzymatic cascades (79, 80). The known UBLs and their putative activating and conjugating enzymes are summarized in Table 2.

Table 2: Known UBLs and their activating and conjugating enzymes

UBL	Identity to ubiquitin (%)	E1 <i>UBL Activating Enzyme</i>	E2 <i>UBL Conjugating Enzyme</i>
Ubiquitin	100	Ube1	Many
FAT10	32	Ube6/Ube1-L2	-
SUMO	18	Uba2/Aos	Ubc9
ISG15	30	Ube1L	UbcH8
NEDD8	55	Uba3	Ubc12
Atg8	-	Atg7	Atg3

(A2-2) Types of Protein Ubiquitination

Mono/Multi-ubiquitination

Proteins that are linked to a single ubiquitin monomer are referred to as mono-ubiquitinated (Illustration 4A). Similarly, addition of ubiquitin monomers on multiple lysine residues of the target protein leads to its multi-ubiquitination or multi-mono-ubiquitination (Illustration 4B). Monoubiquitination has several regulatory roles for the targeted protein, such as inducing changes in subcellular localization, conformation, activity and protein interactions (81). However, this type of ubiquitination does not control active protein turnover or degradation. Protein monoubiquitination also regulates histones and other nuclear proteins in response to DNA-damage (82, 83). Furthermore, the elongation of ubiquitin chains on target proteins to form poly-ubiquitinated proteins requires mono-ubiquitination to proceed as a priming event (84, 85).

Polyubiquitination

Protein polyubiquitination refers to the sequential addition of many ubiquitin monomers to the same protein, thereby forming a chain (Illustration 4C). In contrast to monoubiquitination or multi-ubiquitination, polyubiquitination can attribute various properties to the target proteins. As stated earlier, ubiquitin has 7 lysine residues, each of which can be used to form specific ubiquitin polymers, thereby generating K6-, K11, K27-, K29-, K33-, K48- and K63-linked chains on target protein (Illustration 5). In addition to the role of K48-linked polyubiquitin chains in regulating protein half-life (78, 86), various studies have established emerging roles for the K6-, K11, K27-, K29-, K33- and K63-linked chains in regulating diverse activities of their target protein. These include regulation of DNA repair (87, 88), kinase signaling and activation (89, 90), protein synthesis (91) and protein trafficking (92-94).

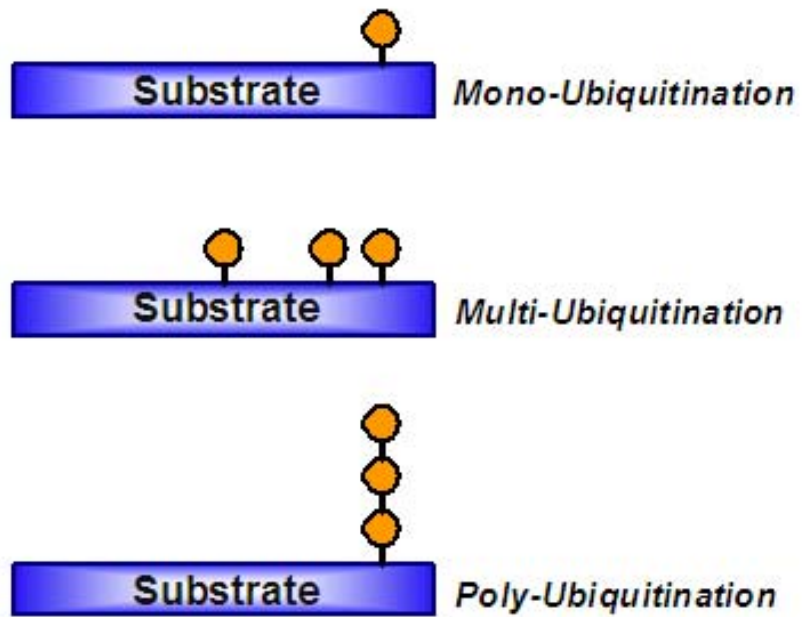


Illustration 4: Types of Ubiquitination.

A common mechanism underlying these functions is that ubiquitin, or polyubiquitin chains, serves as a signal between ubiquitinated proteins and ubiquitin receptors to execute specific biological functions.

K48-Linked Polyubiquitination: It is commonly accepted that formation of K48-linked ubiquitin chains tag the protein for proteasome mediated degradation (78, 86). Recognition and degradation of ubiquitinated proteins by the proteasome requires tetramers or larger oligomers of K48-linked ubiquitin chains on the target proteins (95). In this manner, the ubiquitin-proteasome pathway acts as the principal mechanism for the turnover of cellular proteins in eukaryotic cells (96). This process leads to protein degradation and the recycling of ubiquitin to replenish the cellular pools of monomeric ubiquitin by the action of proteasome-associated enzymes, known as deubiquitinases or DUBs (97).

K63-Linked Polyubiquitination: A vast number of publications support the non-degradative role of K63-linked chains. K63-linked polyubiquitination of the transcription factor Myc leads to the activation of multiple Myc target genes in tumors (98). K63-linked ubiquitination also regulates cellular responses to DNA damage (88). Ubiquitination of histones following DNA-damage by K63-linked ubiquitin polymers was shown to recruit proteins involved in DNA-repair, further strengthening the role of this form of ubiquitination in regulating DNA repair. However, the role of K63-ubiquitination is best studied in cases of cytokine signaling and receptor endocytosis. Many cell-surface receptors are K63-ubiquitinated before their internalization and endosomal sorting (92-94). Many proteins of the TNF- α receptor complex, such as TRAFs (TNF receptor-associated factor), IRAKs (interleukin-1 receptor-associated kinases) and components of the TAK [TGF β (transforming growth factor β)-activated kinase 1]

complex, are targets for K63-polyubiquitination (89) (99, 100). The role of K63-polyubiquitin chains in targeting proteins for proteasomal destruction is not yet fully understood. Recent studies have reported the accumulation of both K48- and K63-linked ubiquitinated proteins after inhibition of the proteasome, suggesting K63-linked proteins may also regulate protein degradation (101).

K11- and K29-Linked Polyubiquitination: In addition to K48-linked ubiquitin chains, atypical polyubiquitin chains linked by K11- and K29-residues have also been reported to degrade tagged proteins. However, these degradation processes are not exclusively proteasomal, and also include lysosomal or endoplasmic reticulum-mediated degradation (102-104).

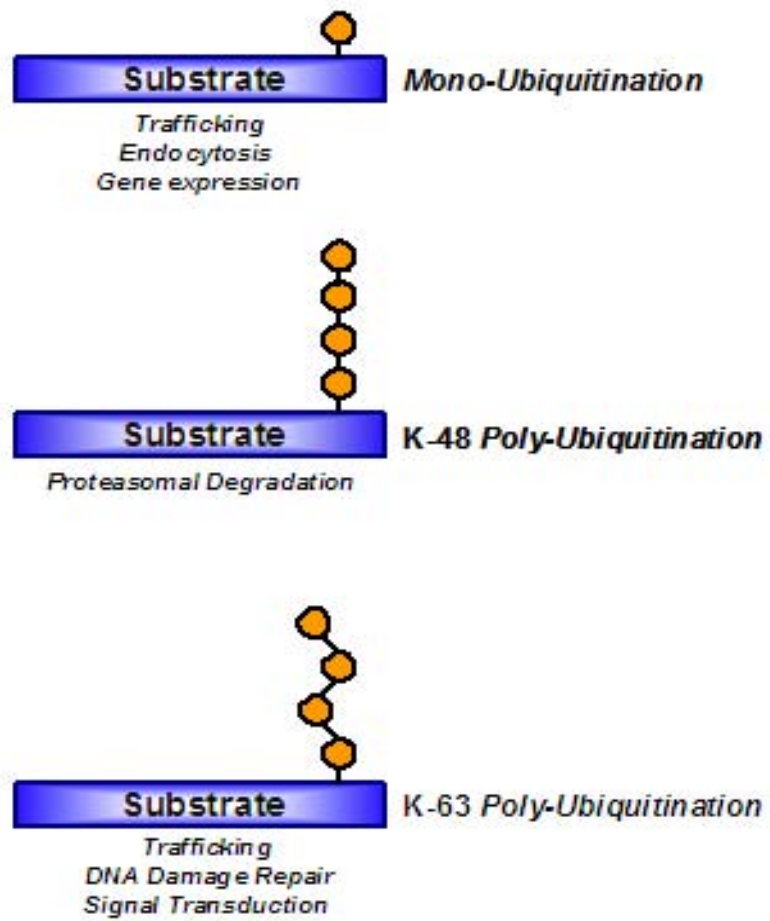


Illustration 5: Role of Lysine-specific polyubiquitin chains.

(A2-3) Enzymes of the Ubiquitination Pathway

(A2-3.1) Ubiquitin Conjugating Enzymes

Ubiquitin Activating Enzyme (UBE/E1)

The transfer of ubiquitin to target protein is mediated in a carefully orchestrated three-step reaction, involving initiating with ubiquitin conjugating enzyme. UBE mediates the first reaction in the ubiquitin pathway by a two step process involving adenylation and thio-ester bond formation. The reaction proceeds with the activation of free ubiquitin using its C-terminal glycine in an ATP-dependent process. This adenylation reaction leads to the formation of ubiquitin adenylate, with the release of PP_i (Illustration 6A, *Step 1*). In the second step, the activated/adenylated- ubiquitin now binds to a cysteine residue of E1 forming a covalent-thioester linkage, with the release of AMP (105, 106) (Illustration 6A, *Step 2*). Subsequently, UBE1 catalyses the adenylation of a second ubiquitin molecule, thereby getting attached to two distinct ubiquitin molecules; one bound by covalent-thioester bond to the catalytic cysteine of the UBE1 enzyme [Ub(T)] and another ubiquitin [Ub(A)] which is associated non-covalently (Illustration 6A, *Step 3*). A previous study showed that a partially purified UBE1-ubiquitin complex containing only the single ubiquitin (T) is capable of transferring the ubiquitin to the next enzyme in the ubiquitination cascade (UBE2), this transfer is accelerated by the presence of ubiquitin(A) or ATP (107). Until recently, it was believed that there exists a single UBE (UBE1) which initiates the ubiquitination pathway. However, recent reports have described the presence of a dual ubiquitin charging/activating system, with the identification of a new UBE called UBA6 or UBE1L2 (108-110).

Ubiquitin Conjugating Enzymes (UBC/E2) and Ubiquitin Ligases (E3)

While the human genome encodes for 2 UBE enzymes, it encodes at least 38 UBCs (111). These enzymes are characterized by the presence of conserved ubiquitin conjugating domains and active cysteine residues which are used to interact with UBEs (112). The binding of E2 to E1 is facilitated when E1 is charged with activated ubiquitin (110). The thioester-linked ubiquitin is transferred from E1 onto the active-site cysteine of an ubiquitin-conjugating enzyme by another thioester bond. Though E1/E2 enzymes for other UBLs have similar structure to the E1/E2 for ubiquitin, E2s for ubiquitin specifically interact with the two E1s of the ubiquitin pathway (110). It is proposed that E2s may participate in the recognition of the protein substrate, either directly or in combination with an E3 enzyme, thereby providing substrate specificity to the whole cascade (78). After being charged with ubiquitin, E2s engage E3 ligases to catalyze substrate ubiquitination. Once bound to substrate protein, the E2–E3 pair catalyzes ubiquitin chain elongation, during which additional ubiquitin molecules are attached to the substrate, leading to its polyubiquitination. The cascade is summarized in Illustration 6B.

Ubiquitin-protein ligases (E3) have central roles in determining the selectivity of target protein to be tagged with ubiquitin (78). A single E2 can interact with several different E3s. Some of the well known E2–E3 pairs include the yeast Skp–cullin–F-box protein (SCF) and the E2 cell division cycle 34 (Cdc34) (113); human anaphase-promoting complex (APC) and the E2 UBE2C (114); the human TNF receptor-associated factor 6 (TRAF6) and the heterodimeric E2 UBC13 (89). Though a single E2 can bind to multiple E3s, the interactions are usually weak (115).

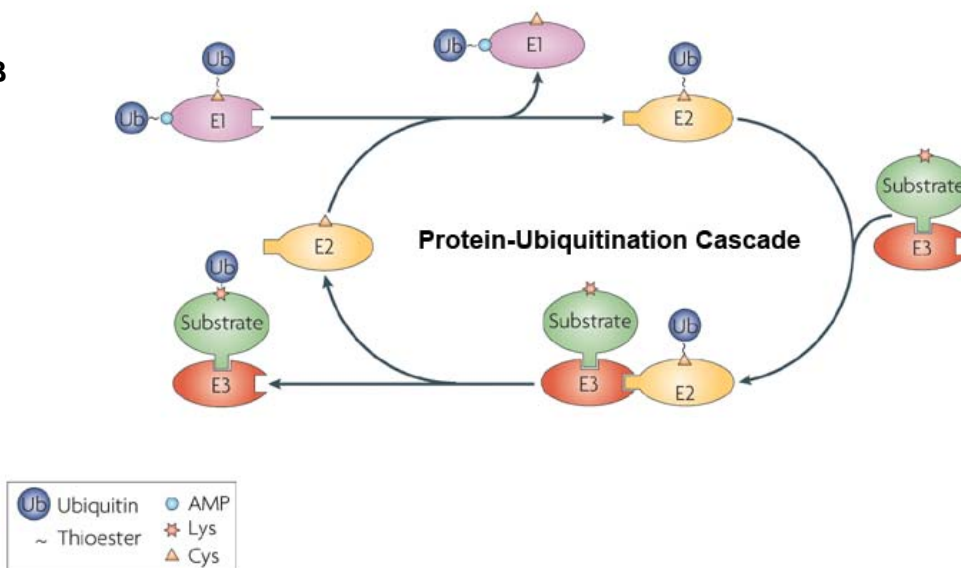
A**B**

Illustration 6: Protein Ubiquitination Cascade.

(A). Ubiquitin activating enzyme (UBE1) activates ubiquitin using ATP via adenylation. The activated ubiquitin is then transferred to the active site of UBE1 and forms a thio-ester bond with active cysteine in the pocket. Concurrently, UBE1 activates another uncharged ubiquitin, thereby getting double-loaded with two ubiquitin molecules.

(B). The activated ubiquitin is transferred to the ubiquitin conjugating enzyme (E2) at its active site. With the help of a ubiquitin ligase (E3), which recruits substrates, E2-E3 complex transfers the ubiquitin to the substrate. This leads to the dissociation of E2 from the E3, allowing it to be recharged with ubiquitin for the next round of transfers.

* Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology 10, 755-764 (November 2009)

Since polyubiquitination can be of different types depending on the type of lysine being used, formation of specific lysine linked polyubiquitin chains is another intrinsic property of many E2s (111). E2 induce changes in the orientation of the acceptor ubiquitin in order to expose the preferred lysine to the active site of E2, thereby controlling the preference for the formation of specific lysine-linked polyubiquitin chains (111, 116, 117).

(A2-3.2) Ubiquitin Deconjugating Enzymes

Protein ubiquitination is a two-way process that can be reversed by deubiquitinase enzymes (DUBs). DUBs recognize ubiquitinated proteins and remove their ubiquitin tags. Just like ubiquitination which regulates protein function, deubiquitination regulates the fate and function of ubiquitin-conjugated proteins. Deubiquitinating enzymes are mainly cysteine proteases (with an exception of few enzymes), constituting the largest family of enzymes in the ubiquitin system, implying their diverse function in the regulation of ubiquitin-mediated pathways. Over 100 proteins with DUB like activity have been identified (118). Apart from removing ubiquitin from protein-ubiquitin conjugates, DUBs also facilitate cleavage of ubiquitin from its precursors and unanchored polyubiquitin chains (119). Thus, DUBs regulate and maintain the homeostasis of free ubiquitin pools in the cell. Apart from to their active-site domains, most DUBs also contain additional domains that regulate substrate recognition and protein-protein interactions (120).

The DUB enzymes are broadly divided into two sub-groups: Ubiquitin C-terminal hydrolase (UCH) and the ubiquitin-specific proteases (USP) (119). USP comprise the largest sub-class of DUBs in human, while only 4 known UCH DUBs have been described (118). UCH-L1/L2/L3 exhibit tissue specificity in terms of protein expression UCH-L1 is localized mainly in brain and testis, while UCH-L3 is present exclusively in

brain, lung and hematopoietic cells (121). DUBs have been shown to be involved in numerous cellular processes, such as growth regulation, gene transcription, cell cycle control (120-122).

(A2-4) Targeting the ubiquitination pathway for therapeutic

(i) Proteasome Inhibitors: The ubiquitin system has emerged as the focus of molecular targeting in developing novel cancer therapeutics. One of the best approaches that have yielded valuable clinical impact is the treatment of multiple myeloma and lymphoma patients with proteasome inhibitors. Although alternations in proteasomal activities have been seen in multiple diseases, no defects have been seen in tumor cells (123). A number of proteasome inhibitors have being developed, such as MG-132, lactacystin, epoxymycin (124). However their clinical utilities are limited by broad specificity and irreversibility. A boronic-acid derivative, bortezomib (Velcade, Millennium Pharmaceuticals), was initially identified in a screening of boronic-acid derivatives, to identify potent anti-proliferative agents. Bortezomib inhibited tumor cell growth at low nM concentrations ($IC_{50} \sim 10$ nM) (125), reversibly inhibiting the active sites in the 20S proteasome (126). Bortezomib-mediated proteasome inhibition promotes tumor cell apoptosis a number of mechanisms, including the induction of endoplasmic-reticulum stress (127). One of the main mediators of apoptosis induction upon proteasome inhibition by bortezomib is the accumulation of p53. The accumulation of p53 further stimulates DNA repair, cell cycle arrest and senescence (128, 129). The increased accumulation of p53 can further induce the expression of its target genes such as p21 and p27, both of which are involved in cell cycle arrest (130). In myeloma cells, bortezomib treatment induces activation of the JNK pathway, which activates caspase-3 to induce apoptosis (131). Additionally, bortezomib treatment leads to release of cytochrome c, resulting in the activation of caspase- 9 (132). Apart from

inducing the apoptotic pathway in myeloma cells, bortezomib also blocks the NF κ B pathway, leading to the suppression of cyclin D1 transcription (133). A summary of key signal transduction and cell cycle regulatory pathways that are controlled via proteasomal activities is shown in Table 3. The mechanism of action of bortezomib and its effect on various cellular processes has been reviewed extensively in (126) (134).

Bortezomib is currently used as a first line of therapy to treat patients with hematological tumors, such as multiple myeloma and relapsed mantle-cell lymphoma (135-137). The success of bortezomib in the treatment of hematological malignancies has led to the analyses of bortezomib on solid tumors. Several studies are currently ongoing to investigate the anti-tumor effects of bortezomib in patients with non-small cell lung carcinoma (NSCLC), breast cancer, head and neck squamous cell carcinoma (HNSCC) and renal cell carcinoma [reviews in (134)]. Although the inhibition of proteasome is expected to confer non-specific effects, proteasome inhibitors are novel and unique in their mechanism of action that preferentially target transformed tumor cells over normal cells (138). Another proteasome inhibitor derived from lactacystin, NPI-0052, is currently undergoing clinical trials and is potentially more promising because of its oral bio-availability (139). These reports suggest that despite little specificity against a component of the ubiquitin system, any perturbations in the machinery render tumor cells much more sensitive to apoptosis as compared to non-transformed cells.

Table 3: Regulation of cellular processes and signaling by proteasome

Process	Proteins degraded by the proteasome	Reference
Cell cycle control	Cyclins A, B, D, E; cyclin-dependent kinase inhibitors (p27, p21 ^{WAF1/CIP1}); cdc25 phosphatase	Adams J; Cancer Cell (2004), Vol 5, Issue 5, pp 417-421
Oncogenic transformation	C-fos, C-jun, N-myc	Adams J; Oncologist (2002), Issue 7 pp. 9-16
Tumor suppression	p53	Adams J; Cancer Cell (2004), Vol 5, Issue 5, pp 417-421
Apoptosis	Bax	Li and Dou; Proc. Natl. Acad. Sci. USA (2000), Issue 97 pp. 3850-3855.
Protein turnover	80% of cellular proteins	Adams J; Cancer Cell (2004), Vol 5, Issue 5, pp 417-421
NF- κ B activation	I κ B	Karin M; Nat. Rev. Cancer (2002), Issue 2, pp. 301-310
Antiapoptotic factors (Bcl-2, IAP, TRAIL)		Almond and Cohen; Leukemia (2002), Vol 16, pp. 433-443
ER stress (URP)		Lee AH; Proc. Natl. Acad. Sci. USA (2003), Issue 100, pp. 9946-9951

(ii) Inhibitors of Ubiquitin Conjugating Enzymes (E1/E3): Since E3 ligases bind directly to target proteins, they are attractive candidates for developing small molecules which disrupt the target protein ubiquitination and thereby its cellular activities. If the target protein happens to be an oncogene, any change in its ubiquitination state by blocking its specific E3 ligases may activate apoptosis. Well-studied E3 enzymes with oncogenic capacity are the RING-type E3 enzyme Hdm2, a crucial negative regulator of the tumor-suppressor protein p53, and the multi-subunit SCF ligases that regulate cell-cycle progression (140-142). Small-molecule inhibitors, such as Nutlins, have been generated that specifically target the E3 ligase activity of Hdm2 (143, 144). Treatment with these inhibitors has been shown to cause accumulation of p53 (similar to that in case with bortezomib), enhancing its signaling activity and inducing apoptosis in a p53-dependent manner. However, since Nutlins block the interaction between wild-type p53 and Hdm2, they can only be used in tumors with wild-type p53. Tumor cells with mutated or deleted p53 are insensitive to Nutlin induced apoptosis (140). An alternative strategy is the development of small molecules which rather bind to the substrate protein and prevent the interactions with E3 ligase/s. RITA is a small molecule that has been shown to bind the N-terminus of p53 and promote growth arrest (145), that is independent of the p53 mutational status.

The inhibitors of apoptosis (IAPs) are a family of proteins which confer anti-apoptotic activity by blocking the proteolytic activity of caspases, thereby promoting cell survival. IAPs are often highly expressed in most tumors, and play a critical role in metastasis (146). IAPs are RING-domain containing E3-ligases, which regulate their own auto-ubiquitination in response to apoptotic stimuli, leading to their proteasomal degradation (147). Under normal conditions, the function of IAPs is counteracted by mitochondrial proteins called SMAC/DIABLO. By binding to IAPs, SMAC allows caspase activation and initiation of apoptosis. Approached to inhibit the activities of

IAPs by SMAC mimetics (148, 149) or anti-sense compounds (150) have been shown to induce cell death.

The role of UBE1 in malignant transformation was recently described by Xu et al (151). The authors showed that ubiquitinated proteins content in malignant cells compared to normal cell. Using a small molecule compound against UBE1, they demonstrated anti-tumor effects in mouse models of leukemia and the induction of ER stress. Prior to this, another group had reported the use of an UBE1 inhibitor which differentially induced apoptosis in tumor cells (152). These findings demonstrate novel mechanisms to induce tumor cell death by inhibiting the UBE1, thereby underscoring its importance in tumor cell survival.

(iii) Inhibitors of Ubiquitin De-conjugating Enzymes (DUBs): DUBs also represent appropriate therapeutic targets as several reports have confirmed a role for these enzymes in the stabilization of proteins involved in transformation, cell cycle regulation, apoptotic protection and drug resistance [reviewed in (120, 122)]. Other studies firmly establish a role for DUBs in a broad spectrum of diseases including cancer (153), viral and bacterial pathogenesis (154, 155) as well as neurodegenerative disorders (156, 157). DUBs such as USP1, USP9x, UCH-L1, USP2a and USP44, have been shown to play a role in tumor cell survival by regulating key oncoproteins, preventing their proteasomal degradation (153, 158, 159). USP1 regulates DNA-repair by inhibiting the monoubiquitination of the Fanconi anemia D2 (FANCD2) and proliferating cell nuclear antigen (PCNA) (160). USP9x has been recently shown to be overexpressed in multiple myeloma patients, supporting tumor cell survival by preventing proteasomal degradation of MCL-1, an anti-apoptotic protein (158). Similarly, USP2a was reported to regulate proteasomal degradation of cyclin D1, thereby supporting tumorigenesis (159). Another deubiquitinase, USP28, is overexpressed in colon and breast tumors, and causes the stabilization of oncoproteins such as cyclin E1 and c-Myc (161, 162).

UCH-L1 has been linked to Parkinson's disease (163). These observations suggest that silencing of specific DUBs in tumor cells may be safe and useful for therapy of oncogene addicted cells. Due to the specialized role of DUBs in the ubiquitin cycle and their emerging role in control of multiple signaling pathways and oncoproteins, DUB inhibitors may be useful anti-cancer agents (164).

Peptide based potent, irreversible inhibitors of DUBs, such as ubiquitin aldehyde and ubiquitin vinyl sulfone (UbVS) have been previously described (165, 166). However, their therapeutic potential is limited by their high mol wt and limited cellular bioavailability. The first small molecule reported with anti-DUB activity was D12-prostaglandin J2, which was initially shown to inhibit cellular DUBs, leading to massive accumulation of ubiquitinated proteins ($IC_{50} \sim 30 \mu M$). However, the profile of specific DUBs affected by this compounds has not yet been described (167). Treatment of lung cancer cells with small-molecule inhibitors targeting the UCH-L1 has shown some success (168).

B. Materials and Methods

B1. Cell culture, Chemical Reagents and Enzymes

Human multiple myeloma MM1.S and mantle cell lymphoma Z138 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Adherent cell lines such as human embryonic kidney 293T (HEK293T) cells were cultivated on Dulbecco modified essential medium (DMEM) containing 10% FBS. All cells were cultured and maintained at 37°C in a humidified atmosphere. RPMI-1640 and DMEM were purchased from HyClone (Thermo Fisher Scientific, Waltham, MA).

To establish a Flag-tagged Jak2 overexpressing cell line to study protein-protein interactions of Jak2, HEK293 cells were transfected with p3xFLAG-CMV™-10 vector containing Flag-Jak2 cloned in-frame with the 3X-Flag peptide (see details below). The transfected cells were then grown for 2-weeks in the presence of antibiotic selection medium (DMEM + 10% FBS + 1.5 mg/ml G418). The surviving cells were further pooled grown to higher density and frozen in liquid nitrogen till use. The expression of Flag-Jak2 was confirmed by probing the lysates of these cells with either anti-Flag antibody or anti-Jak2. Flag-Jak2 overexpressing HEK293 cells were cultured in DMEM, supplemented with 10% FBS and G418 (1 mg/ml) at 37°C in humidified conditions.

WP1130 and AG490 were provided by Dr. William Bornmann (University of Texas, M.D. Anderson Cancer Center, Houston, TX). Other reagents used in this study were obtained from the following sources: Bortezomib (Millennium Pharmaceuticals); Mini-Complete and PhosSTOP inhibitory cocktails (Roche Applied Science, Indianapolis, IN); Ub-AMC, Suc-LLVY-AMC, Boc-LRR-AMC, 20S human proteasome, Hemagglutinin-tagged ubiquitin vinyl methyl sulfone (HA-UbVs), UCH-L1, UCH-L3, USP5 (BostonBiochem, Cambridge, MA).

B2. Plasmids and siRNA transfection

cDNA template from MM1.S cells was used to amplify full length human Jak2 using specific 5' and 3' primers containing *NotI* restriction sites. To generate a Flag-tagged Jak2 expression plasmid, the amplified Jak2 PCR product was cloned in-frame into p3xFLAG-CMV™-10 (Sigma Aldrich, St. Louis, MO). Jak2 was sequenced using internal primers to confirm the wild-type status of the cloned product. Expression plasmids for HA-tagged ubiquitin (WT/48Only/63Only) were kindly provided by Dr. Bryant Darnay (University of Texas, MD Anderson Cancer Center). HEK293T cells (10^5 /ml per well) were seeded into 12-well tissue culture plates (~50% confluence). Vectors expressing Flag-Jak2 or variants of HA-Ubiquitin were transfected using Eugene HD (Roche).

ON-TARGETplus siRNA (Thermo-scientific-Dharmacon) for USP9x and USP5 (30 nM each) were used to knockout USP9x and USP5 in HEK293T cells using Lipofectamine 2000 (Invitrogen). Protein levels were estimated after 72 hours of transfection by western blotting.

B3. Lysate Preparation, Immunoprecipitation and Western Blotting

(i) **Detergent-Soluble Lysate:** To prepare detergent soluble and insoluble fractions, cells were lysed in cold isotonic lysis buffer A [10 mM Tris-HCl (pH 7.5), 0.5 % Triton X-100, 150 mM NaCl along with Mini-Complete and PhosSTOP] for 15 min on ice and centrifuged 10 min at 20,000 RCF. The clarified supernatant was used as a source of the detergent soluble cell fraction.

(ii) **Detergent-Insoluble Lysate:** The residual pellet was sonicated in equal volumes of boiling 1x Laemmli reducing sample buffer to extract the detergent insoluble proteins.

(iii) Whole Cell Lysate: Whole cell lysates were prepared by boiling and sonicating the cell pellets in 1x Laemmli reducing sample buffer.

(iv) Immunoprecipitation: To immunoprecipitate polyubiquitinated Jak2, cells were lysed in 100 μ L denaturing buffer (1%SDS, 250 mM NaCl) and incubated at 65°C for 30 min to disrupt protein-protein interactions. The lysate was sonicated further to dissolve proteins in the insoluble pellet into the denaturing buffer (to ensure solubility of the total cell lysate). The lysates were spun at 20,000 RCF for additional 10 mins. The clarified supernatant was then diluted by addition of 900 μ L lysis buffer A. Immunoprecipitation was carried out with either anti-Jak2 or anti-Flag antibody at 4 °C for 2-4 h with rotation, followed by overnight incubation with protein-A/G sepharose beads to pull-down immune-complexes. The beads were washed three times in the isotonic buffer and boiled in 1x reducing Laemmli buffer.

(v) Co-Immunoprecipitation: To investigate the association of Jak2 with other proteins after WP1130 treatment, co-immunoprecipitation studies were performed. WP1130 incubation makes Jak2 accumulate in the detergent-insoluble fraction in a time-dependent manner, making it difficult to immunoprecipitate Jak2 without disrupting protein-protein interactions. Therefore, only a brief incubation with WP1130 (1 hr) was given in co-immunoprecipitation studies, to allow maximum recovery of Jak2 from the detergent-soluble fraction. Flag-Jak2 HEK293 cells or Z138 cells were either treated with WP1130 (5 μ M) or DMSO for 1 hour, followed by their lysis in ice-cold lysis buffer A. The soluble fraction from these cells was used to immunoprecipitate Jak2 by anti-Flag or anti-Jak2 antibodies. The association between Jak2 and components of aggresome was ascertained by immunoblotting with anti-HDAC6, anti-HSP90.

B4. Antibodies

Antibodies used in this study were purchased from following sources: anti-actin, anti-Flag (Sigma-Aldrich, St. Louis, MO); anti-ubiquitin clone P4D1, anti-HDAC6, anti-HSP90, anti-20S proteasome, anti-pJak2 (Y1007/1008), anti-Jak2, goat anti-rabbit/mouse/rat IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA); anti-flotillin1, anti-Jak1, anti-Tyk2 (BD Biosciences, San Jose, CA); anti-Jak2, anti-pStat3, anti-Stat3, anti-PARP, anti-SOCS1, anti-MCL1 (Cell signaling Technology, Danvers, MA); anti-ubiquitin clone FK1 (Enzo Life Sciences International, Plymouth Meeting, PA); anti-HA clone 3F10 (Roche Applied Science Indianapolis, IN); anti-HDAC6, anti-USP5, anti-USP9x, anti-Jak2 (Bethyl Labs, Montgomery, TX).

B5. 20S Proteasome Activity Assay

Fluorogenic substrate Suc-LLVY-AMC was used to assay for chymotryptic-like activity of the 20S proteasome. To assay for *in vivo* proteasome inhibition, Z138 cells were treated with WP1130 (5 μ M) or MG132 (5 μ M) for 2 hours. The cells were lysed in ice-cold lysis buffer (50 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl & 1% Triton X-100) and their lysates clarified by centrifugation at 20,000 RCF for 10 min. Equal amounts of protein from each sample were then incubated at 37°C with 100 μ M fluorogenic substrate.

To assay for direct inhibition of the 20S proteasome *in vitro*, purified 20S human proteasome (200 ng, Boston Biochem) was incubated with WP1130 (5 μ M) and MG132 (5 μ M) for 30 min at 37°C before addition of the substrates. Fluorescence intensity was measured using a spectrophotometer at excitation 360 nm & emission 460 nm. Assays were performed in triplicate, and statistical significance was determined with a paired Student's *t* test.

B6. Confocal Microscopy

HEK293T cells treated with WP1130 or DMSO for 4 hours were washed twice in PBS, followed by fixation using 4% formaldehyde for 15 min. The cells were permeabilized in 0.5% Triton X-100 for 5 min. Slides were then incubated in blocking solution (5% goat serum) for 1 h at room temperature. Incubation with the primary antibodies (1:100) was carried out overnight at 4°C, and the slides were washed three times with 0.2% Triton X-100/PBS buffer. Alexa-Fluor anti-mouse and Alexa-Fluor anti-rabbit immunoglobulin antibodies were used as secondary antibodies. The slides were washed three times and stained for nucleus detection with Hoechst 33342. Images were acquired using an Olympus FluoView™ 500 (Tokyo, Japan). Images represent grouped Z-stacks (Z=0.5 µm) from each sample.

B7. Sub-cellular Fractionation

Z138 cells were either treated with 5 µM WP1130 or DMSO for 2 hours, followed by their lysis and processing to isolate the cytosolic, membrane, nuclear and cytoskeletal fractions. The ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, San Diego, CA) was used to extract each fraction according to the manufacturer's instruction. Markers for each fraction were included to assess the purity of each fraction.

B8. *In Vitro* Deubiquitination Assays

(i) Ub-AMC Protease Assay: Cells were lysed in ice cold DUB buffer containing 50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 5 mM MgCl₂, 150 mM NaCl and 1 mM phenylmethylsulfonylfluoride. Briefly, 5 µg of clarified lysate from untreated, WP1130 or bortezomib treated cells were incubated with 500 nM Ub-AMC in a 100 µl reaction volume at 37°C and the release of AMC fluorescence per minute was recorded.

Purified DUBs at optimal concentrations (USP5; 20 nM, UCH-L1; 20 nM, UCH-L3; 5 nM, USP9x; immunoprecipitated from 500 µg Z138 cell lysate) were incubated in DUB buffer containing WP1130 or DMSO (v/v) in a 100 µl reaction volume for 30 min. The reaction was initiated by the addition of 500 nM Ub-AMC and the release of AMC-fluorescence was recorded at ex/em 380/480 using a spectrofluorometer.

(ii) Ub Chain Disassembly: *In vitro* disassembly of purified polyubiquitin chains (K-48/K-63 linked) was performed as described earlier (169). Five µg of lysate from untreated and WP1130 treated Z138 cells prepared in DUB buffer were incubated with K48- or K-63 linked chains (1 µg) for 5, 10 and 15 min at 37°C. The extent of chain disassembly was assessed by western blotting.

B9. Deubiquitinase Labeling Assays

To assay for changes in activity of cellular deubiquitinase enzymes, Z138 and HEK293 cells were lysed in DUB buffer (50 mM Tris pH 7.4, 5 mM MgCl₂, 150 mM NaCl) for 10 min at 4°C. The lysates were centrifuged at 20,000 RFC for 10 min and the supernatant was used for DUB labeling. Equal amounts of lysate were incubated with 500 ng of HA-UbVs for 1 hr at room temperature, followed by boiling in reducing sample buffer and resolving by SDS-PAGE. After protein transfer to nitrocellulose membranes, HA immunoblotting was used to detect DUB labeling.

B10. Measurement of Reactive Oxygen-Species (ROS)

Z138 cells were treated with DMSO, WP1130 and H₂O₂ (positive control) for 1 hour. The cells were washed with PBS and incubated with H₂DCFDA (10 µM) in a CO₂ incubator at 37 °C for 15 min. The DCF fluorescence was measured at 0, 30, 60 and 120 min, respectively, using with excitation and emission wavelengths of 485 nm and

530 nm, respectively. Intracellular ROS accumulation is relative to the fluorescent activity of H₂DCFDA conversion.

B11. Cell Proliferation/Survival Assessment by MTT

Cells were seeded in a 96-well plate at 5,000 cells per well in the presence of increasing concentration of WP1130 for 3 days in a CO₂ incubator at 37 °C. 20 µl of 5 g/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well for 2 h at 37°C. The cells were then lysed in 10% SDS-buffer and absorbance at 570 nm relative to a reference wavelength of 630 nm was determined with a microplate reader. The concentrations resulting in 50% inhibition of cell growth (IC₅₀ values) were calculated.

B12. Statistical analysis

A statistical analysis was performed using two-tailed Student's *t*-test to assess the statistical significance of treated groups versus control groups. The results with *P*-values of less than 0.05 were considered to be statistically significant.

C. Results

C1. Small-Molecule Inhibitors of the Jak2/Stat3 Pathway

Using cell based screening assays, we screened a small chemical library (>200) of AG490 structural analogues to identify more potent antagonists of the Jak2/Stat3 pathway. This approach involved incubation (2 hr) of MM1.S cells with the test compound, followed by a brief IL-6 (15 min) stimulation to activate the Jak2/Stat3 cascade. Potency of the compounds was then judged by comparing the levels of pStat3 in IL-6 stimulated cells against the cells pre-treated with the inhibitor. As shown in Figure 1 (upper panel), initial studies led to the identification of more effective AG490 derivatives (AG1801, AG2019), which suppressed Stat3 activation at lower μM concentrations (12-25 μM). A second round of screening using AG1801 analogues led to the identification of WP compounds; namely WP1066, WP1129 and WP1130. These 2nd generation AG490 derivatives displayed even more potent inhibitory activities against the Jak2/Stat3 pathway (Figure 1, lower panel).

We found WP1130 to be the most potent analogue among the newer derivatives, completely suppressing IL-6 driven phosphorylation of Stat3 at much lower doses (2.5-5 μM) than AG490 itself (Figure 2A). However, WP1130 showed no inhibition of Jak2 autophosphorylation in an *in-vitro* kinase assay even at higher μM concentrations (Figure 2B). These results suggest that in contrast to its parental tyrphostin AG490, WP1130 potently inhibits Jak2 driven signaling without directly affecting its kinase activity.

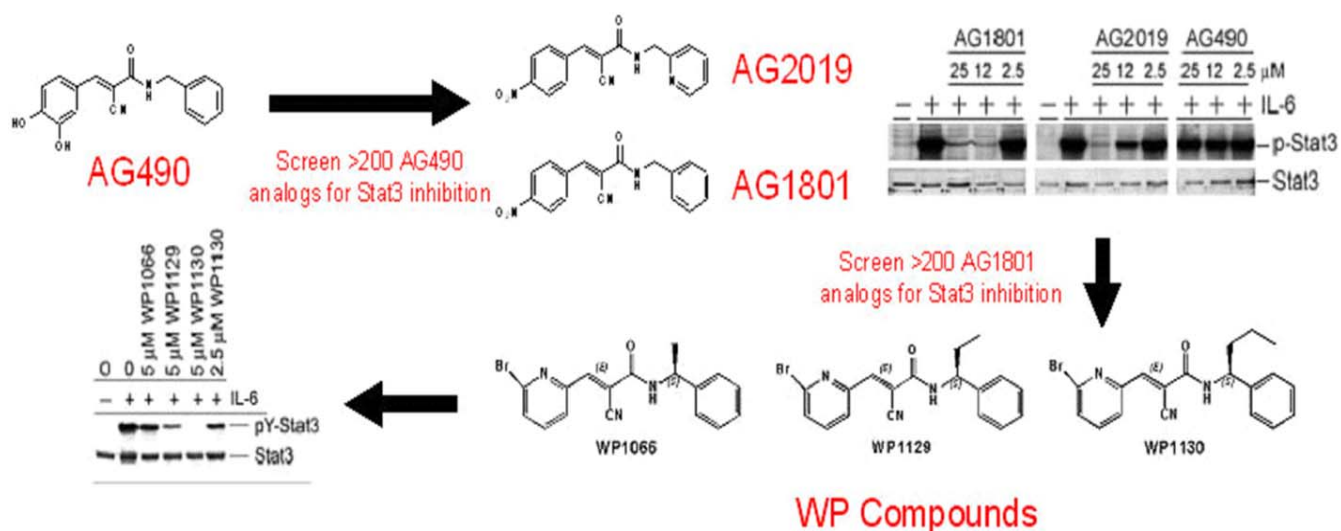


Figure 1. Development of potent inhibitors of Jak-Stat signaling

Cell based assay were used to identify AG490 derivatives which effectively suppress IL-6 stimulated Stat3 activation. MM1s cells were pre-treated for two hours with the indicated concentration of first (AG1801, 2019) and second generation (WP1066, 1130) AG490 analogues, followed by a brief IL-6 stimulation (10 ng/ml, 15 min). The extent of suppression of Jak-Stat signaling was assessed by monitoring the phosphorylation of Stat3.

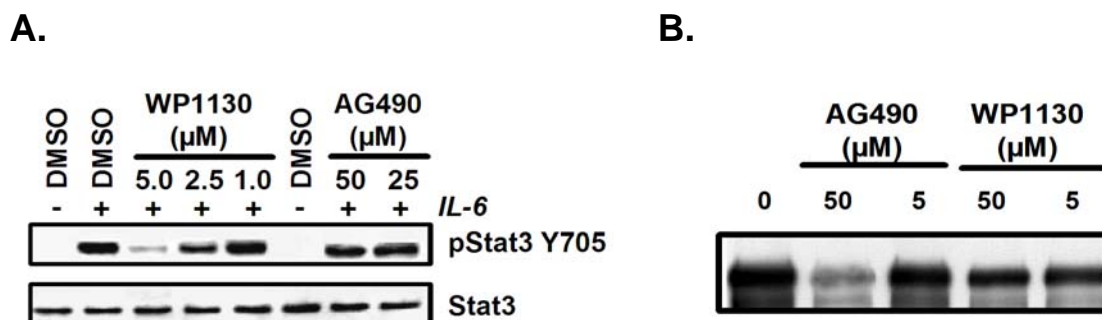


Figure 2. Inhibition of Jak2-Stat3 signaling by WP1130

(A). MM1s cells were treated with indicated concentration of WP1130 or AG490 for two hours and stimulated with IL-6 to activate Jak2-Stat3 signaling. WP1130 effectively suppresses Stat3 activation at low μM concentration compared to AG490.

(B). Purified Jak2 was incubated in the presence of WP1130 or AG490 for 2 hours in kinase buffer. The autophosphorylation of Jak2 was assessed by incubation in labeling reagent consisting of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM Na₂VO₄, and 5 μCi ³²P-ATP. After 30 min at room temperature, proteins were resolved by SDS-PAGE and phosphorylation detected by autoradiography.

C2. Anti-Tumor Properties of WP1130

Our preliminary studies showed that mantle cell lymphoma Z138 and multiple myeloma MM1s cells displayed high apoptotic sensitivity to WP1130 ($IC_{50} \sim 1 \mu M$). WP1130 effectively suppressed the growth of various other cancer cell lines of lymphoid and myeloid origin at low μM concentration (Figure 3A). Treatment of Z138 cells with WP1130 induced rapid apoptosis, evident by the appearance of cleaved PARP after 2-4 hours of treatment. Direct comparison of WP1130 with AG490 or bortezomib, which is clinically active in mantle cell tumors, illustrated distinctions in the apoptotic onset and activity of each compound (Figure 3B). Similar results were observed in MM1s and HEK293 cells, suggesting WP1130 has potent anti-tumor properties (Figure 3C). The anti-proliferative and apoptotic effects of WP1130 and less active derivatives (WP1066, WP1034) against various tumors have recently been reported in CML (73), melanoma (74), glioblastoma (75) and myeloproliferative disorders (76).

C3. Suppression of Jak2 Signaling by WP1130

Since we observed potent inhibition of Jak2/Stat3 signaling by WP1130 in the absence of Jak2 kinase inhibition, we investigated the mechanism of WP1130-mediated suppression of the Jak2-Stat3 pathway. We immunoprecipitated Jak2 from the soluble extracts of DMSO, IL-6 and WP1130/IL-6 treated MM1.S cells. Immunoblotting with anti-phosphotyrosine revealed that WP1130 pre-treatment inhibited IL-6 driven Jak2's phosphorylation. Interestingly, probing the same membrane for Jak2 showed a decline in Jak2 levels in WP1130 treated cells (Figure 4). The observed down-regulation of Jak2 in WP1130 treated cells was an unexpected result. Loss of Jak2 protein by WP1130 treatment may be responsible for the suppression of Stat3 activation.

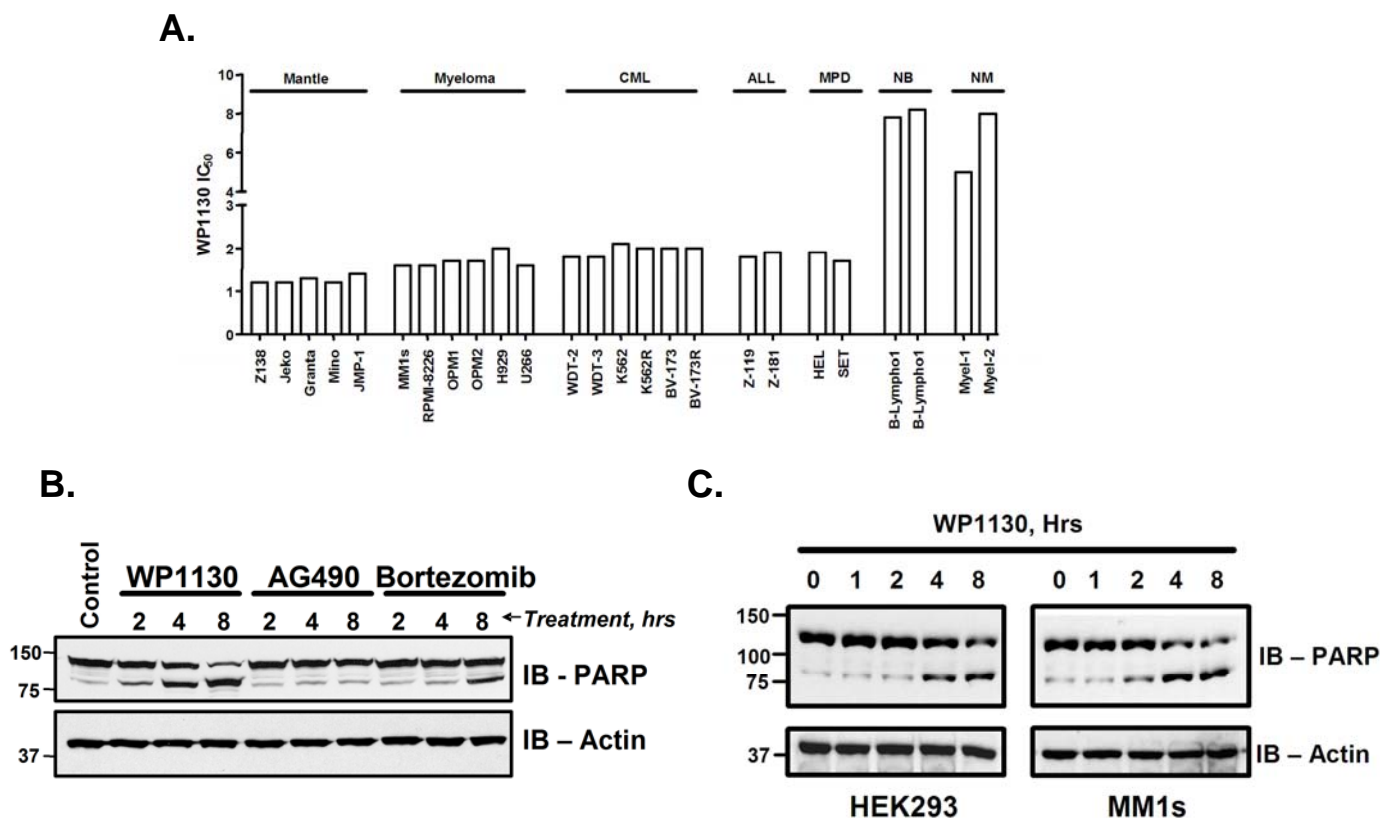


Figure 3. Anti-proliferative effects and induction of apoptosis by WP1130 in various cell lines of lymphoid and myeloid origin.

(A). IC₅₀ values of WP1130 in various cell lines; CML= chronic myelogenous leukemia, ALL= acute lymphocytic leukemia, MPD= myeloproliferative disorders, NB= normal B-cells, NM= normal myeloid cells.

(B). Mantle cell lymphoma (Z138) cells were treated with WP1130 (5 μ M), AG490 (100 μ M) or Bortezomib (50 nM) for the interval indicated. Whole cell extracts were probed for detection of cleaved PARP as an indicator for onset of apoptosis. Actin was probed on the same blot as a protein loading control.

(C). MM.1S and HEK293T cells were treated with 5 μ M WP1130 for the indicated intervals. Whole cell extracts were probed for detection of cleaved PARP as an indicator for the onset of apoptosis. Actin was probed on the same blot as a protein loading control.

To investigate whether AG490 has similar effects on Jak2 protein levels, we treated MM1.S cells with DMSO, WP1130 (5 μ M) or AG490 (50 μ M) for 2 hours, followed by their lysis. Jak2 was immunoprecipitated from soluble lysates and subjected to immunoblotting. We noted that Jak2 down-regulation was a unique characteristic of WP1130, which was not exhibited by AG490 (Figure 5A), suggesting different mechanisms for inhibition of Jak2-Stat3 signaling. To further examine the kinetics of WP1130 mediated Jak2 down-regulation, we treated MM1.S cells with 5 μ M WP1130 for 30, 60 and 120 min. The cells were then briefly stimulated with IL-6 to activate Jak2-Stat3 signaling. Shorter intervals of incubation of MM1.S cells with 5 μ M WP1130 (30 min-120 min) showed a rapid, time dependent, loss of Jak2 protein from cell lysates (Figure 5B). The decline in Jak2 protein levels paralleled the loss of downstream Stat3 activation upon IL-6 stimulation; however, no decline in Stat3 protein was observed (Figure 5B). These results show that WP1130 induces down-regulation/degradation of Jak2, leading to the suppression of downstream Stat3 signaling. These results are aligned with previous reports regarding Jak2 down-regulation in cells treated with WP1130 and the related compound WP1066 (73, 76, 77).

We next examined whether a proteolytic pathway was activated by WP1130 to induce Jak2 down-regulation. We assessed inhibitors of the proteasomal [MG-132] and other proteolytic pathways [lysosomes (NH_4Cl), cysteine proteases (E64D), papain/trypsin (Antipain), chymotrypsin (TPCK)] for their ability to suppress WP1130 mediated Jak2 down-regulation. MM1s cells were incubated with effective concentrations of each proteolytic inhibitor for 1 hr, prior to treatment with 5 μ M WP1130 for additional 2 hours. The cells were then stimulated with IL-6 to activate the Jak2 signaling cascade. We observed no inhibition of WP1130-mediated Jak2 down-regulation in the presence of any of these protease inhibitors (Figure 6), and Stat3 phosphorylation remained suppressed by WP1130.

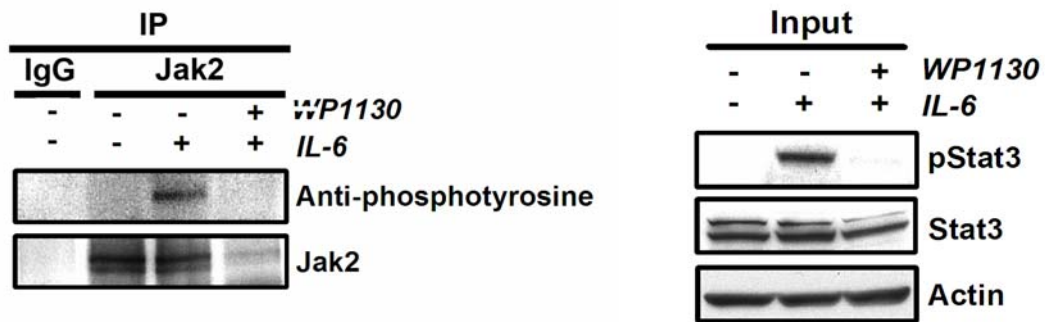
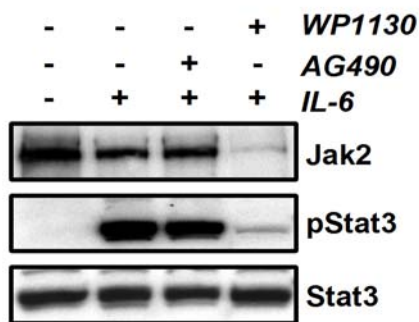
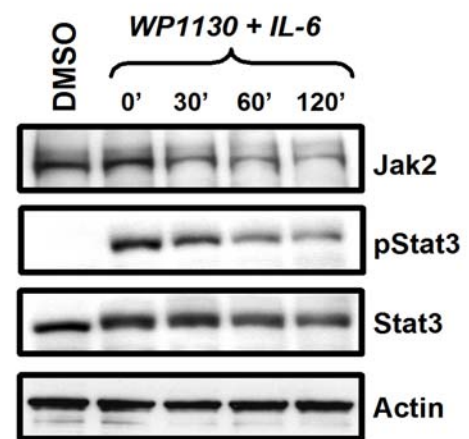


Figure 4. WP1130 induces rapid down-regulation of Jak2

MM1s cells were treated with WP1130 (5 μ M) for 2 hours, followed by brief IL-6 stimulation. Untreated or IL-6 alone treated cells served as controls. Jak2 was immunoprecipitated from the lysates (supernatant) and probed for its phosphorylation and residual protein levels using anti-phosphotyrosine (4G10) and anti-Jak2 antibody. WP1130 treatment inhibits Jak2 phosphorylation by decreasing its protein levels. A fraction of the lysate (input) from these cells was also probed to confirm the inhibition of Stat3 activation by WP1130. No loss in Stat3 protein levels was detected upon WP1130 treatment.

A.**B.****Figure 5. Kinetics of WP1130 mediated down-regulation of Jak2**

(A). MM1s cells were treated with WP1130 (5 μ M) or AG490 (100 μ M) for two hours and stimulated with IL-6. The supernatant lysates were used to detect changes in Jak2 protein levels upon WP1130 and AG490 treatment. While WP1130 effectively suppressed Stat3 activation through potent Jak2 down-regulation, AG490 had no effect on Jak2 protein levels. Untreated or IL-6 alone stimulated cells served as controls. Stat3 was blotting was used as protein loading control.

(B). MM1.S cells were incubated with 5 μ M WP1130 for the indicated time interval, followed by IL-6 stimulation. The lysates were probed to assess the kinetics of Jak2 down-regulation and loss of Stat3 activation.

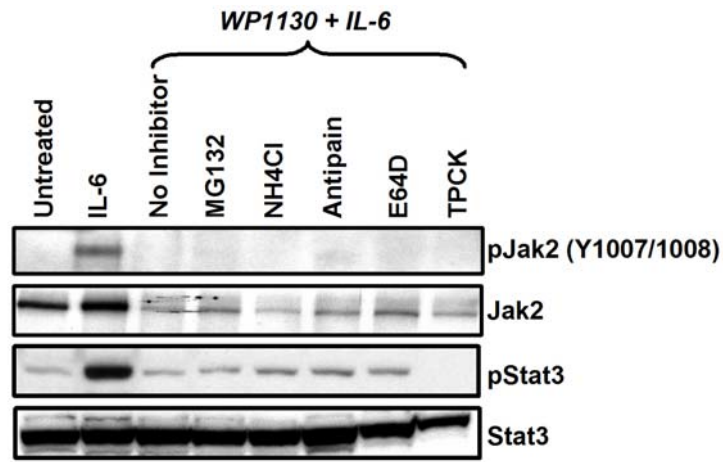


Figure 6. Effect of protease inhibitors on WP1130-induced Jak2 down-regulation.

Inhibitors of various proteolytic pathways were used to prevent WP1130 induced Jak2 down-regulation. MM1s cells were pretreated with MG-132 (5 μ M), NH₄Cl (10 mM), Antipain, E64D and TPCK for 1 hour. The cells were then further incubated with WP1130 (5 μ M) for an additional 2 hour, followed by IL-6 stimulation. The lysates were probed for any inhibition in the loss of Jak2 and the restoration of Stat3 signaling in the presence of inhibitors. DMSO, IL-6 alone and WP1130 alone treated cells served as experimental controls.

C4. WP1130 Mediated Trafficking of Jak2

To investigate potential accumulation of Jak2 into other cellular fractions that are not solubilized by standard extraction conditions, we modified our lysate extraction protocol to investigate the possible accumulation of Jak2 into the detergent insoluble cell fraction (see materials and method). Examination of the detergent-soluble extracts (soluble) of WP1130 treated Z138 cells demonstrated a rapid loss of Jak2 protein, as noted in earlier experiments. However, we observed a subsequent time-dependent increase in Jak2 levels in the detergent-insoluble fraction (insoluble) of the cell, with no apparent decline in Jak2 protein from total cell extracts (Figure 7A). Careful examination of the lysate preparation protocols of earlier reports describing Jak2 down-regulation (73, 76) by WP1130/WP1066 revealed the use of soluble supernatants as the source of lysate. Our results show that WP1130 treatment induces re-localization of Jak2 into the insoluble fraction, without inducing its degradation. These results are consistent with our protease/proteasomal inhibitor studies which failed to block the loss of Jak2 protein in WP1130 treated cells.

Among the Jak family proteins, Jak2 was found to be the most sensitive to WP1130 treatment. Treatment of Z138 cells with 5 μ M WP1130 for 2 hours showed very little impact on the accumulation of Tyk2 and Jak1 in the insoluble fraction (Figure 7B); consistent with a previous report where WP1066 showed no change in their protein levels (77). Cytokine receptor was also not affected by WP1130 as no accumulation of gp130 was seen in the insoluble fraction of WP1130 treated Z138 cells. Furthermore, treatment of Flag-Jak2 overexpressing HEK293 cells with WP1130 showed a similar accumulation of Flag-tagged Jak2 in the detergent insoluble fraction following WP1130 treatment (Figure 7C).

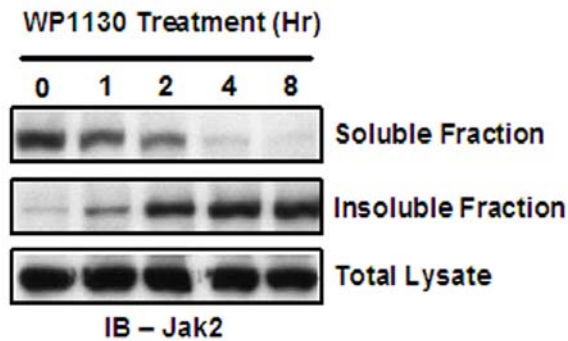
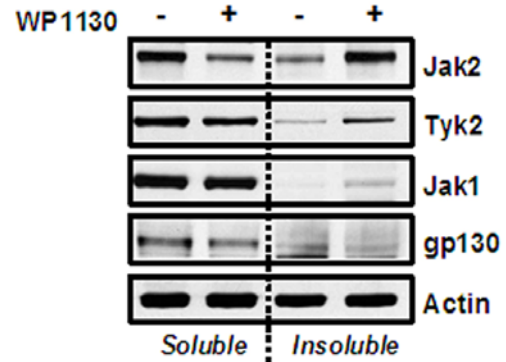
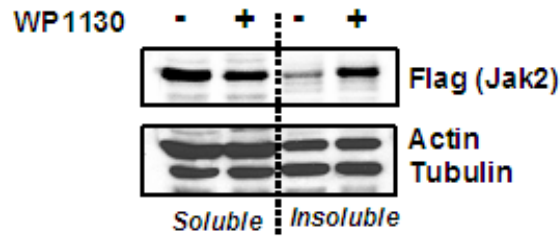
A.**B.****C.**

Figure 7. Effects of WP1130 on trafficking of Jak2 and Jak-family members.

(A). Z138 cells were treated with 5 μ M WP1130 for indicated time period. The cells were then processed to extract their detergent-soluble, detergent-insoluble or total cell lysate as described in the materials and methods. The presence of Jak2 in each fraction was assayed by immunoblotting with anti-Jak2 antibody.

(B). Z138 cells were treated with DMSO (-) or WP1130 (+) and their soluble and insoluble fractions were analyzed to assess the specificity of WP1130 towards other Jak family members (Jak1, Tyk2) and gp130. Actin was used as a loading control.

(C). Flag-Jak2 overexpressing HEK293 cells were treated with WP1130 (+) or DMSO (-) for 2 hours, followed by the extraction of soluble and insoluble lysates. The lysates were probed with anti-Flag to detect Jak2 and Actin/Tubulin was used as loading controls.

D.

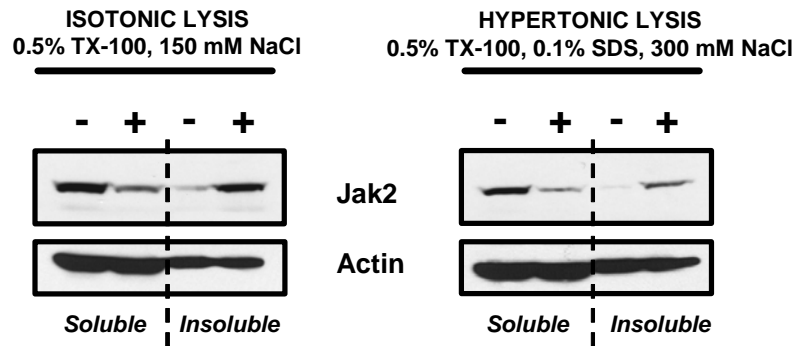


Figure 7. Effects of WP1130 on trafficking of Jak2 and Jak-family members (continued).

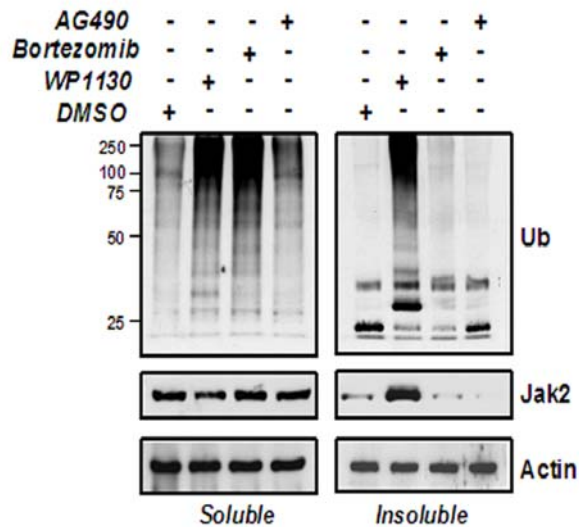
(D). Z138 cells were treated with DMSO (-) or WP1130 (+) for 2 hours. The cells were then split and lysed under isotonic conditions or hypertonic/stringent conditions. The detergent-soluble and -insoluble lysates obtained from each lysis condition were then probed for the presence of Jak2. Actin was used as a loading control.

Furthermore, the accumulation of Jak2 in the detergent-insoluble fraction of WP1130 treated cells was seen under both isotonic/mild and hypertonic/stringent lysis conditions (Figure 7D). This suggested that the observed accumulation of Jak2 in the insoluble fractions of WP1130 treated cells is not merely due to inefficient extraction of the cellular lysates.

C5. Effect of WP1130 on the Accumulation of Ubiquitinated Proteins

Molecular analysis revealed some structural and chemical similarities between WP1130 and a few compounds with described DUB inhibitory activity, such as dibenzylideneacetone (DBA) and curcumin (167). These observations prompted a detailed analysis of effects of WP1130 on cellular protein ubiquitination. Since ubiquitination has been reported to regulate protein accumulation in the detergent-insoluble fraction (170), we examined whether WP1130 affected ubiquitination of cellular proteins, leading to their accumulation in the insoluble fraction. We treated Z138 cells with WP1130, AG490 or bortezomib and probed the detergent-soluble and – insoluble cell fraction for the presence of ubiquitinated proteins. We observed a massive increase in the presence of polyubiquitinated proteins in WP1130 treated cells, resembling bortezomib treatment. However, the increased accumulation of ubiquitinated proteins in the insoluble fraction of WP1130 treated cells was markedly distinct from that of bortezomib treatment (Figure 8A). Additionally, only WP1130 treatment induced Jak2 to accumulate in the insoluble fraction. No changes in either cellular ubiquitinated protein levels or in the distribution of Jak2 were observed in AG490 treated cells. Although Bortezomib treatment led to the accumulation of ubiquitinated proteins in the soluble fraction, no such accumulation was observed in the detergent-insoluble fraction. We further analyzed whole cell extracts (WCE) from WP1130 treated Z138 cells and observed a marked and concentration-dependent accumulation of ubiquitinated proteins

A.



B.

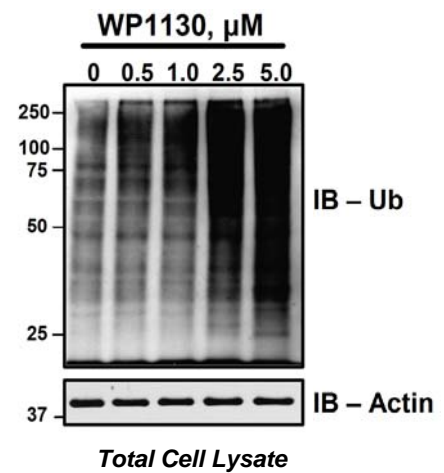


Figure 8. Effects of WP1130, Bortezomib and AG490 on protein ubiquitination and trafficking.

(A). Z138 cells were treated with 5 μ M WP1130, 50 nM bortezomib or 100 μ M AG490 for 2 hours and their lysates were resolved into a detergent-soluble and –insoluble fraction. Changes in the distribution of Jak2 and ubiquitinated proteins by each compound were assessed by western blotting. Actin blotting served as protein loading control.

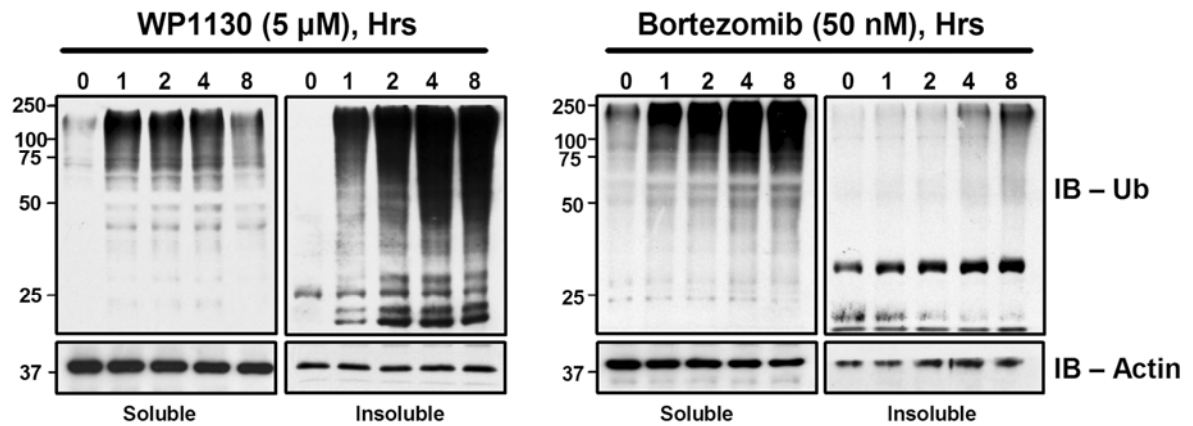
(B). Z138 cells were incubated with the indicated concentration of WP1130 for 2 hrs before whole cell lysates were subjected to immunoblotting with anti-ubiquitin. Membranes were probed for actin as a protein loading control.

(Figure 8B). A similar increase in protein ubiquitination was seen in other WP1130 treated cells including K562, MM.1S, HeLa and HEK293 (data not shown). These results suggest distinctions in the molecular mechanism of WP1130 compared to the proteasome inhibitor bortezomib and AG490.

C6. WP1130 Does Not Act as a Proteasome Inhibitor

To further differentiate between bortezomib and WP1130, we examined the kinetics of accumulation of ubiquitinated proteins into the detergent-soluble and – insoluble fraction. We observed a time-dependent accumulation of ubiquitinated proteins into both the detergent soluble and insoluble fraction of Z138 cells upon treatment with WP1130. Bortezomib (50 nM) did not induce significant accumulation of insoluble ubiquitinated proteins, with only minimal ubiquitin content change in the detergent insoluble fraction even after 8 hours of treatment (Figure 9A). The accumulation of ubiquitinated proteins can occur following proteasome inhibition. Therefore, we next examined whether WP1130 had any effects on 20S proteasome activity in treated cells or against the purified 20S proteasome. As shown in Figure 9B, WP1130 incubation (5 μ M, 2 hr) caused no significant decline in proteasome chymotryptic-like activity *in vivo* (p-value > 0.07) and *in vitro* (p-value > 0.78) while MG-132 (known proteasome inhibitor) substantially inhibited proteasome activity in either assay.

A.



B.

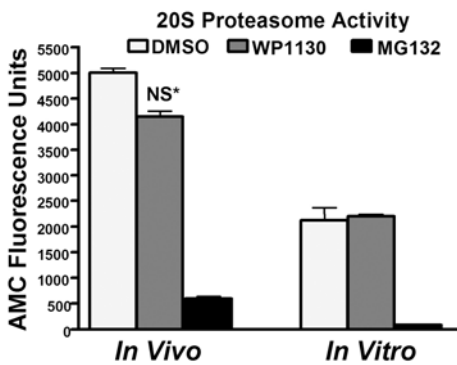


Figure 9. Differential effects of WP1130 and Bortezomib on protein ubiquitination.

(A). Z138 cells were treated with either WP1130 or Bortezomib for the interval indicated. Lysates were separated into detergent soluble and insoluble fractions as described in the materials and methods and probed for the distribution of ubiquitinated proteins and actin as a protein loading control by immunoblotting.

(B). Left (*In vivo*)- Lysates from DMSO (control), WP1130 or MG-132 treated cells (5 μ M, 2 hr) were analyzed for proteasome activity as described the material and methods. **Right (*In vitro*)** - Purified 20S proteasome was incubated with 5 μ M WP1130, 5 μ M MG-132 or DMSO for 30 min before analysis of chymotryptic or tryptic proteasome activities by monitoring substrate cleavage. The results represent the average \pm S.D. from triplicate samples.

C7. WP1130 Induces Trafficking of Jak2 into Aggresomes

Insoluble, ubiquitinated proteins have been previously shown to accumulate in structures called aggresomes (171, 172). Aggresomes, often characterized by the presence of multiple marker proteins such as HDAC6, 20S Proteasome, HSP90/70 along with ubiquitinated proteins, are distinct in their peri-nuclear localization (173, 174). Ubiquitinated proteins are transported to the aggresome by HDAC6 using dynein motors via the microtubule network (175). To further define the Jak2 enriched insoluble fraction, we performed sub-cellular fractionation of WP1130 treated Z138 cells. Following WP1130 treatment, Jak2 was predominantly detected in the cellular cytoskeletal fraction, along with other markers of aggresomes (Figure 10A). To validate the association of Jak2 with the aggresomal machinery consisting of HDAC6, HSP90 and CHIP, we performed co-immunoprecipitation studies. Z138 cells were treated with WP1130 for 30 min to induce Jak2 ubiquitination and facilitate further protein-protein interaction. The detergent-soluble fraction obtained from these cells was used to pull-down Jak2 and probe for associated aggresomal proteins. As shown in Figure 10B, increased association between Jak2, HSP90, CHIP and HDAC6 was noted following brief WP1130 treatment, suggesting ubiquitinated Jak2 was affiliated with aggresome-marker proteins. We further performed confocal microscopy of HEK293 cells treated with DMSO or WP1130 for 2 hours to firmly establish localization of Jak2 in peri-nuclear aggresomes. As shown in Figures 11, we observed accumulation of HDAC6 in dense, compact regions outside the nucleus, co-localizing with ubiquitinated proteins, a hallmark of aggresome formation. We also observed co-localization between ubiquitinated proteins and 20S proteasome (Figure 12) or Jak2 (Figure 13).

A.

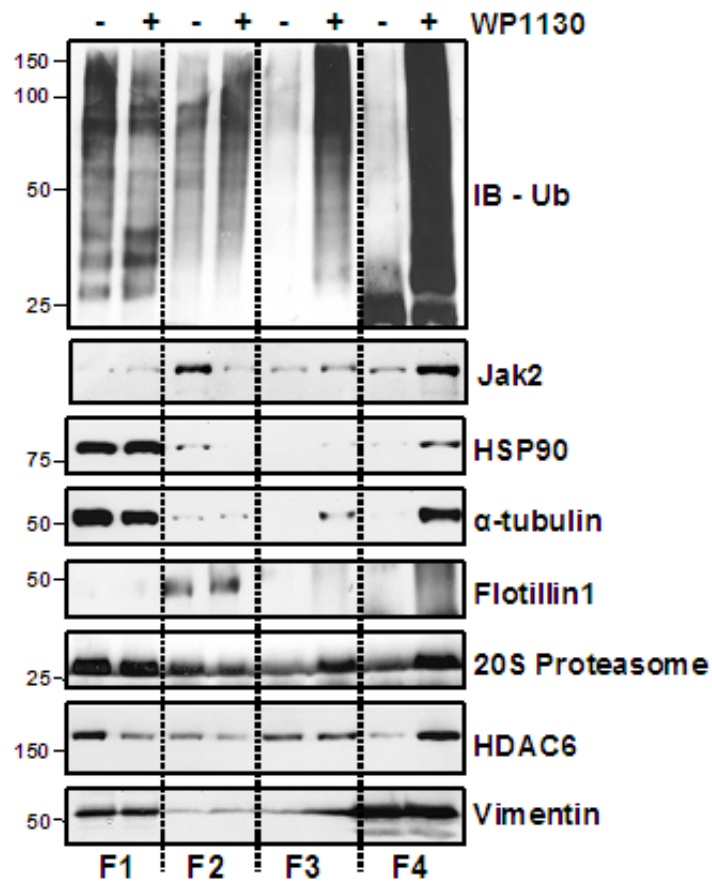


Figure 10. Accumulation of Jak2 in cellular cytoskeletal/Aggresomal fraction.

(A). Z138 cells were treated with DMSO (-) or 5 μ M WP1130 (+) for 2 hrs before cell extracts were subjected to fractionation into cytoplasmic (F1), membrane-associated (F2), nuclear (F3) or cytoskeletal (F4) components. Equal protein content was subjected to immunoblotting for ubiquitin (top) or markers for each fraction. The cytoskeletal fraction from WP1130 treated cells was highly enriched with ubiquitinated proteins as well as Jak2 and aggresome markers.

B.

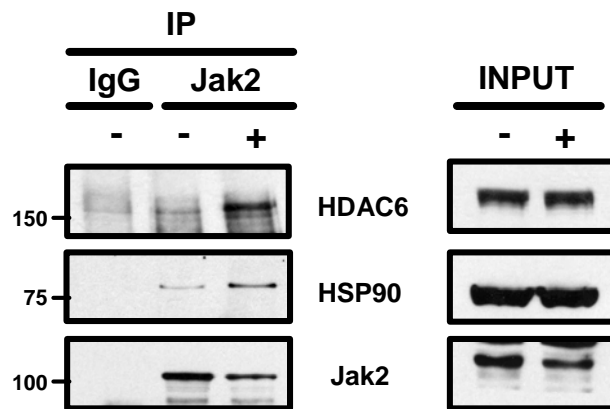


Figure 10. Accumulation of Jak2 in the cytoskeletal/aggresomal cell fraction.

(B). Z138 cells were treated with DMSO (-) or 5 μ M WP1130 (+) for 1 hr before lysis in isotonic lysis buffer. Jak2 was immunoprecipitated as described in the materials and methods to detect its association with components of the aggresome (HDAC6 and HSP90).

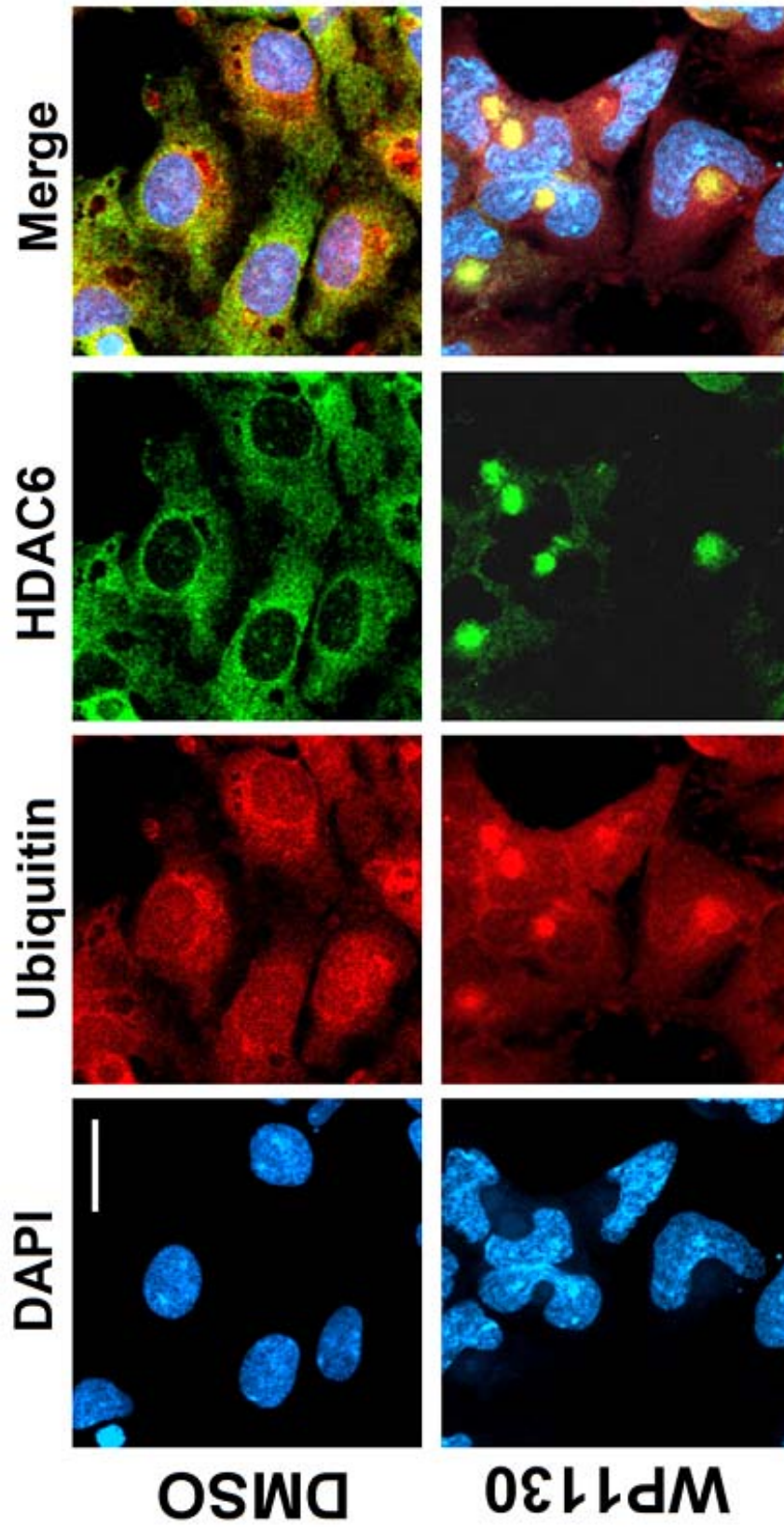


Figure 11. WP1130 induces Aggresome formation.

DMSO or WP1130 (5 μM, 4 hr) treated HEK293T cells were processed for immunofluorescence as described in the materials and method. Treatment with WP1130 led to perinuclear accumulation of aggresome markers such as ubiquitin (red) and HDAC6 (green). Images were acquired using Olympus FluoView™ 500 confocal microscope using 60X water immersion lens and 2X digital zoom (120X total magnification).

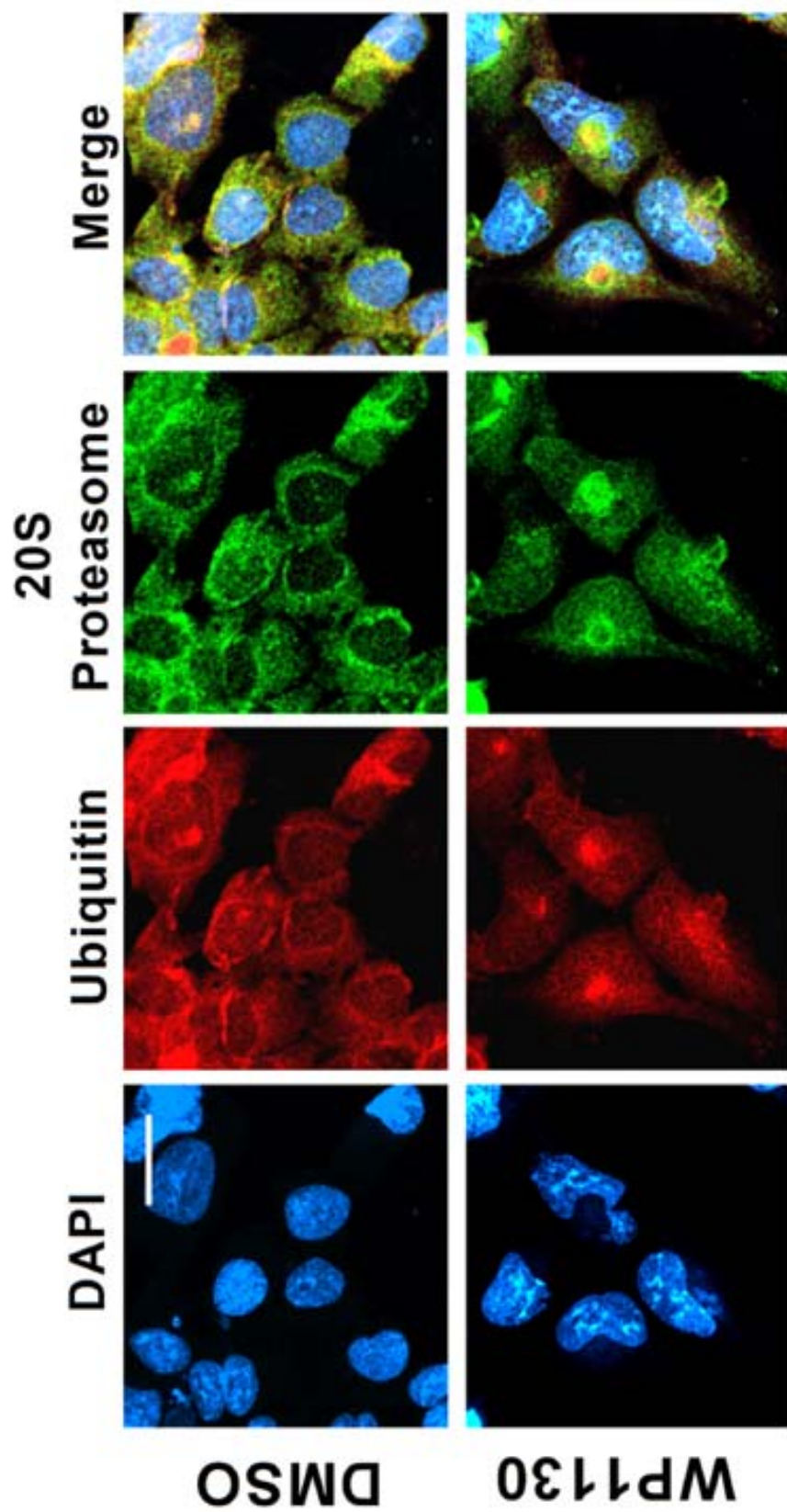


Figure 12. WP1130 induces Aggresome formation

DMSO or WP1130 (5 μ M, 4 hr) treated HEK293T cells were processed for immunofluorescence as described in the materials and method. Treatment with WP1130 led to perinuclear accumulation of aggresome markers such as ubiquitin (red) and 20S proteasome (green). Images were acquired using OlympusFluoView™ 500 confocal microscope using 60X water immersion lens and 2X digital zoom (120X total magnification).

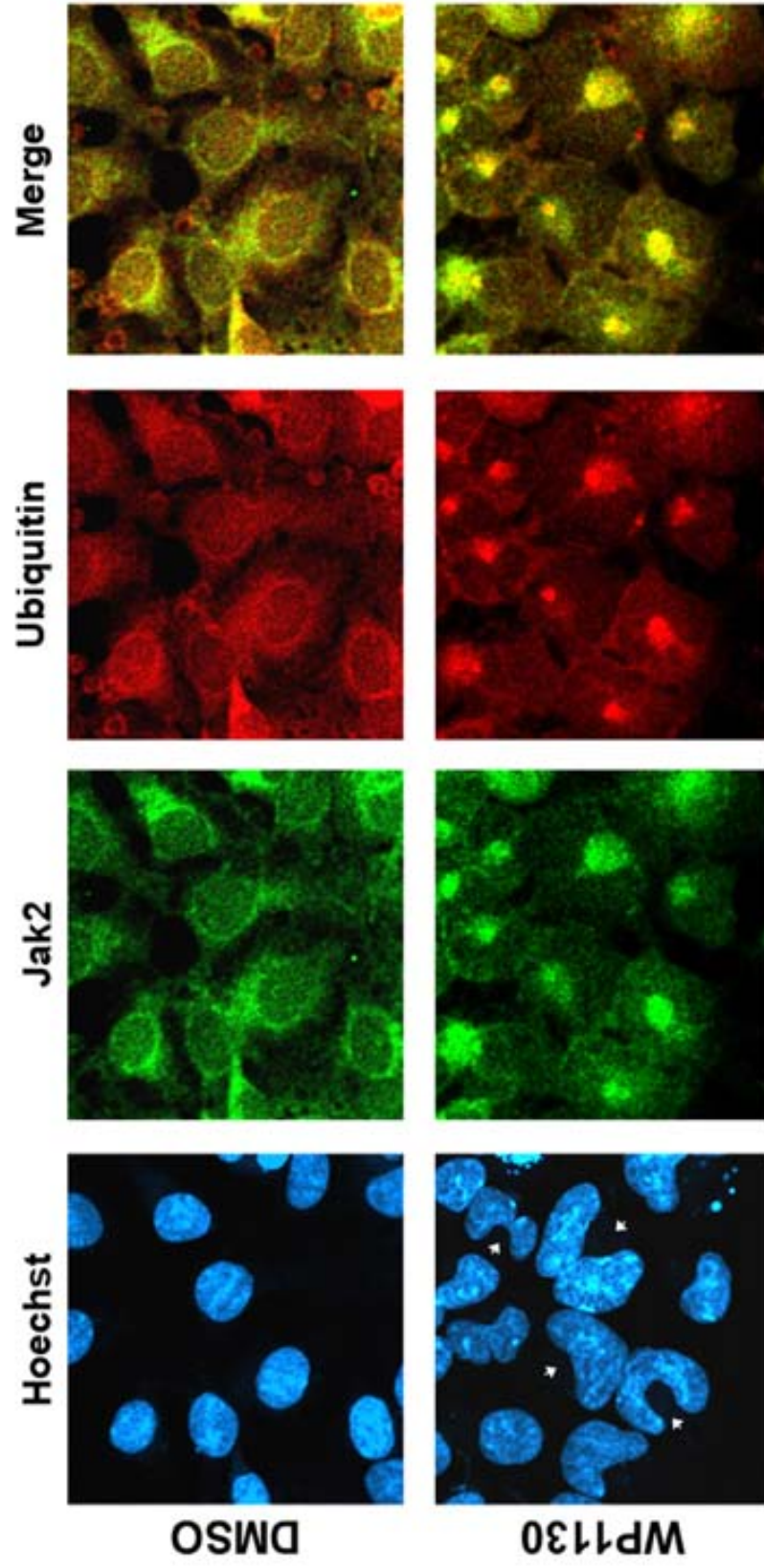


Figure 13. WP1130 induces Aggresome formation .

DMSO or WP1130 (5 μ M, 4 hr) treated HEK293T cells were processed for immunofluorescence as described in the materials and method. Treatment with WP1130 led to perinuclear accumulation of aggresome markers such as ubiquitin (red) and Jak2 (green). Images were acquired using Olympus Fluoview™ 500 confocal microscope using 60X water immersion lens and 2X digital zoom (120X total magnification).

C8. Polyubiquitination of Jak2 by WP1130

Considering the effect of WP1130 on protein ubiquitination, we investigated whether Jak2 undergoes ubiquitination in response to WP1130. Since WP1130 treatment renders Jak2 in-extractable, we used denaturing conditions to immunoprecipitate Jak2 to detect its ubiquitination (see materials and methods). Through this approach we detected rapid ubiquitination of Jak2 following WP1130 treatment. Similar results were obtained in HEK293 cells over-expressing Flag-tagged Jak2 (Figure 14A). Proteasome inhibitor MG-132 was used as a positive control to allow accumulation of endogenously ubiquitinated Jak2. The presence of ubiquitinated Jak2 in WP1130 treated cells was novel and in contrast to its previously described mode of ubiquitination which is dependent on cytokine stimulation (38). Additionally, to differentiate between poly-ubiquitinated Jak2 and its multi-ubiquitinated forms, we used a monoclonal anti-ubiquitin antibody (FK1) that specifically recognizes only poly-ubiquitinated proteins and not their mono-/multi-ubiquitinated forms (81). Immunoblotting with FK1-anti-ubiquitin confirmed the polyubiquitination of Jak2 after WP1130 treatment (Figure 14B).

To further investigate the type of polyubiquitin linkage formed on Jak2 after WP1130 treatment, we used HA-tagged variants of ubiquitin. The ubiquitin variants can either use any available lysine on ubiquitin (WT), or use Lysine-48 only (K48O) or Lysine-63 only (K63O) to form polyubiquitin chains. Flag-Jak2 HEK293 cells were transfected with each HA-Ub variant expression plasmid. Following 2 days of transfection, the cells were then treated with 5 μ M WP1130 for 1 hour, followed by their lysis to immunoprecipitate Jak2 with anti-Flag antibody. Immunoblotting with HA showed the presence of ubiquitinated Jak2 after WP1130 incubation in cells transfected with either WT or K63O variants of HA-ubiquitin. No accumulation of polyubiquitinated Jak2 containing K48-linked chains was observed after WP1130 treatment.

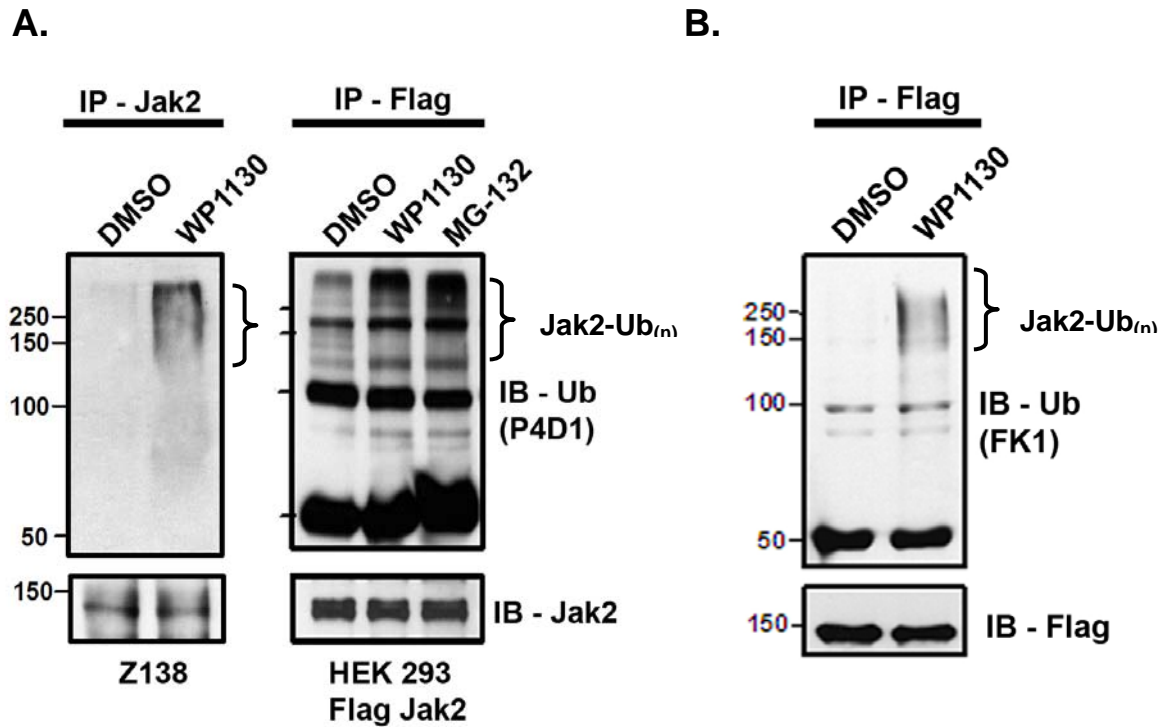


Figure 14. WP1130 induced ubiquitination of Jak2.

(A). Z138 or HEK293 Flag-Jak2 cells were treated with WP1130 (5 μ M) or MG-132 (5 μ M) for 1 hours and lysed under denaturing conditions to immunoprecipitate ubiquitinated Jak2 (as described in materials and methods).

(B). Ubiquitination of Jak2 was probed using 2 different anti-ubiquitin antibodies – P4D1 (detects mono-/multi-/poly-ubiquitinated proteins) and FK1 (detects polyubiquitinated proteins only).

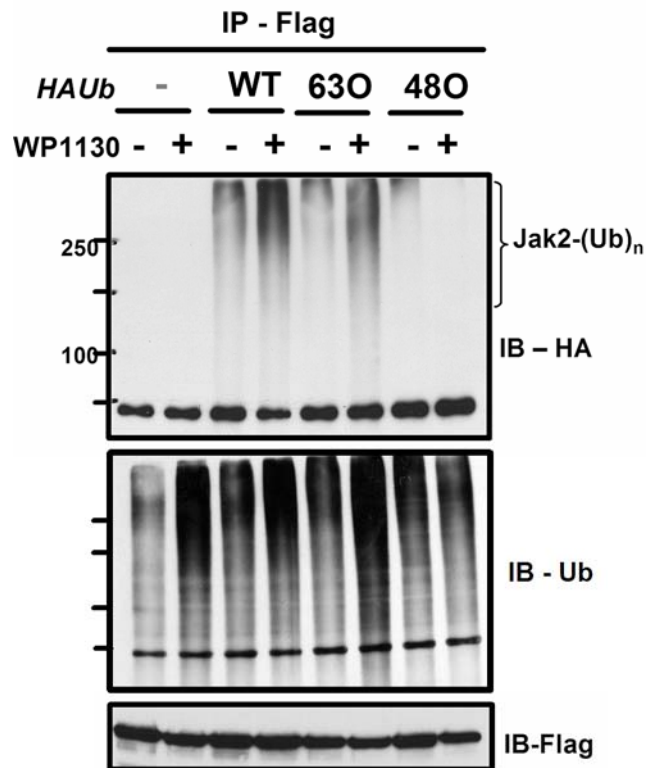


Figure 15. Ubiquitination of Jak2 using K63-Linked polyubiquitin chains

Flag-Jak2 HEK293 cells were transfected with vectors expressing either WT or mutated variants (lysine 63 only/lysine 48 only) of HA-tagged ubiquitin. After 2 days of transfection, the cells were treated with WP1130 (5 μ M) for 1 hour and Jak2 was immunoprecipitated under denaturing conditions using anti-Flag antibody. Jak2 ubiquitination was probed by HA immunoblotting.

C9. WP1130 acts as Deubiquitinase Enzyme Inhibitor

Inhibition of cellular deubiquitinases could lead to an increase in high molecular weight ubiquitinated proteins in the absence of proteasome inhibition (176, 177). As described earlier, WP1130 shares some structural and chemical resemblance to known DUB inhibitors. Therefore, we assessed possible indications of DUB inhibition in WP1130 treated cells. We observed a rapid depletion of monomeric ubiquitin (Figure 16A) and a subsequent increase in the levels of unanchored/free polyubiquitin chains (Ub₄₋₅) in cells treated with WP1130 (Figure 16B). In contrast, bortezomib treatment did not affect the level of free ubiquitin or unanchored ubiquitin chains. This observation suggests that WP1130 may reduce ubiquitin recycling and amass ubiquitinated proteins through inhibition of deubiquitinase activity. To directly assess the impact of WP1130 on DUB activity in treated cells, cell lysates from control and treated cells were incubated with ubiquitin-AMC and fluorescence generated as a consequence of substrate cleavage was measured as an indicator of DUB activity. Treatment with WP1130 significantly reduced DUB activity by nearly 50% by 4 hours (p- value 0.0095). Interestingly, we did not see any change in DUB activity in bortezomib treated cells (Figure 16C).

We also examined the effect of WP1130 treatment on *in vitro* deubiquitination/disassembly of purified K48-linked or K63-linked polyubiquitin chains. Lysates from WP1130 treated (2 hr) or untreated cells were incubated with 1 µg of unanchored polyubiquitin chains for 5, 10 and 15 min at 37°C. Lysates from untreated cells caused an almost complete disassembly of polyubiquitin chains in contrast to the limited disassembly observed with lysates from WP1130 treated cells (Figure 16D). Lack of chain disassembly of both K48- and K63-linked polyubiquitin chains from the lysates of WP1130 treated cells is in agreement with our previous observations of increased accumulation of both types of ubiquitin linkages upon WP1130 treatment

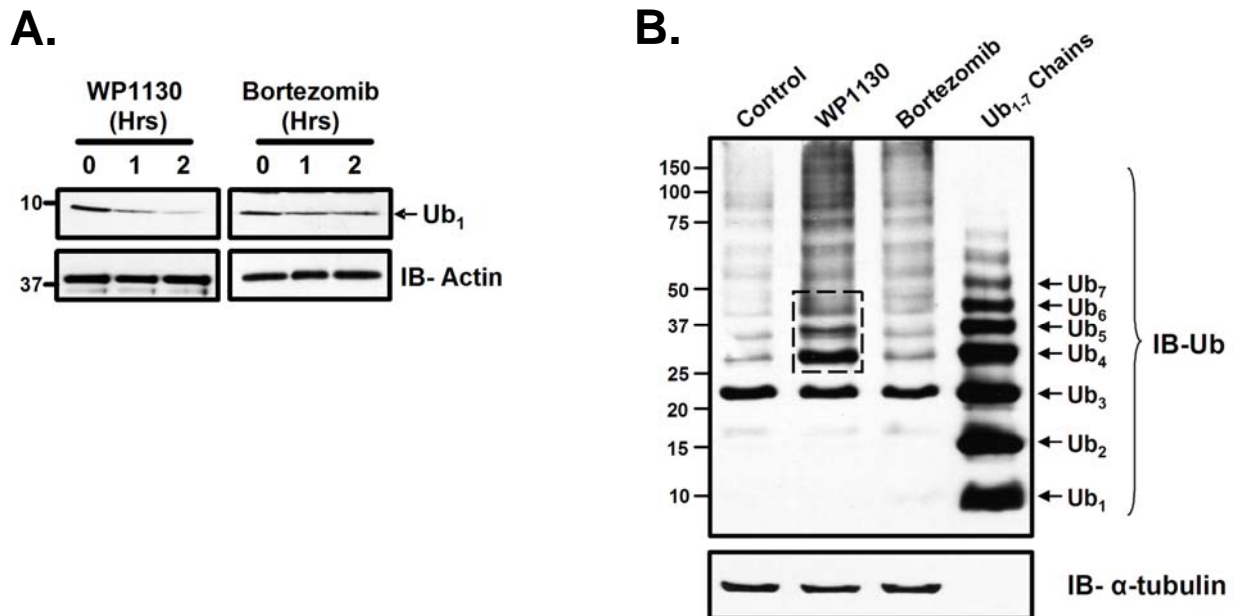
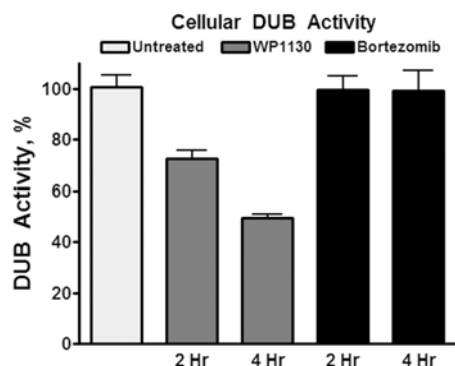


Figure 16. WP1130 reduces cellular deubiquitinating activity.

(A). Z138 cells were incubated with DMSO, WP1130 (5 μ M) or bortezomib (50 nM) for the indicated intervals. Whole cell extracts were probed for the levels of mono-ubiquitin by running high percent (15%) gels.

(B). Z138 cells were treated with DMSO (Control), 5 μ M WP1130 or 50 nM bortezomib for 2 hrs before whole cell lysates were resolved on high percent cross-linked gels and subjected to immunoblotting for ubiquitin and actin. Preformed ubiquitin polymers (Ub₁₋₇) were loaded in the last lane as a marker for the relative migration of individual ubiquitin chains.

C.



D.

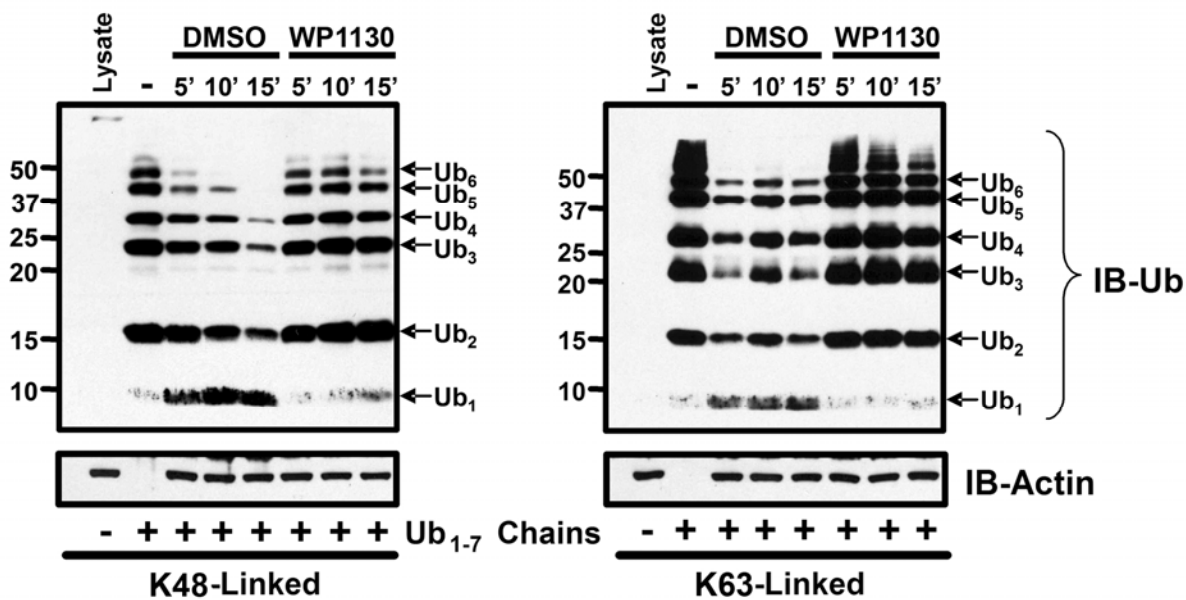


Figure 16. WP1130 reduces cellular deubiquitinating activity (continued).

(C). Z138 cells were treated with 5 μ M WP1130, 50 nM bortezomib or DMSO for the interval indicated before extracts (5 μ g) were prepared as described in the materials and methods and incubated with fluorogenic substrate. The results are representative of one analysis performed in triplicate. Similar results were obtained in two additional independent assays.

(D). Lysates (5 μ g) from vehicle treated (DMSO) or WP1130 treated (5 μ M, 4 hrs) cells were incubated with 1 μ g of K-48 linked (left) or K-63 linked (right) free chains of polyubiquitin (Ub₁₋₇) for 5, 10 and 15 min at 37°C. The extent of free chain hydrolysis in each lysate was examined by western blotting. Actin was blotted as a protein loading control.

(Figure 16D). These results suggest that WP1130 treatment inhibits cellular DUB enzyme/s required for the breakdown of both K48- and K63- specific ubiquitin linkages.

C10. DUB Inhibitory Profile of WP1130

To further assess DUB inhibition in WP1130 treated cells, we employed a technique described by Borodovsky et al (166, 178) to identify the profile of active cellular DUB activity in intact cells. Hemagglutinin-tagged ubiquitin vinyl methyl sulfone (HA-UbVs) acts as a DUB suicide substrate, forming a covalent adduct with active DUB enzymes. Specific cellular DUBs can be identified by anti-HA blotting, as previously shown in various cell types (178-180). Changes in specific DUB activities are measurable by monitoring HA-labeling in lysates from control and treated cells. A dose-dependent and time reduction in the labeling of DUBs corresponding to USP9x, USP5, USP14 and UCH37 was seen in cells treated with WP1130 (Figure 17A, 17B). In contrast, no change in DUB labeling was noted in cells treated with AG490, confirming distinctions in the activity and mechanism of action of the parental tyrphostin and WP1130. An *in vitro* analysis using cell lysates was performed to investigate direct DUB inhibition by WP1130. Briefly, untreated Z138 lysates were incubated with 5 μ M WP1130 or DMSO for one hour at 37°C, followed by labeling with HA-UbVs. Incubation of cell lysate with WP1130 showed a reduction of HA-labeling of the same DUBs as those noted in intact cells (Figure 17C), suggesting that WP1130 caused direct DUB inhibition.

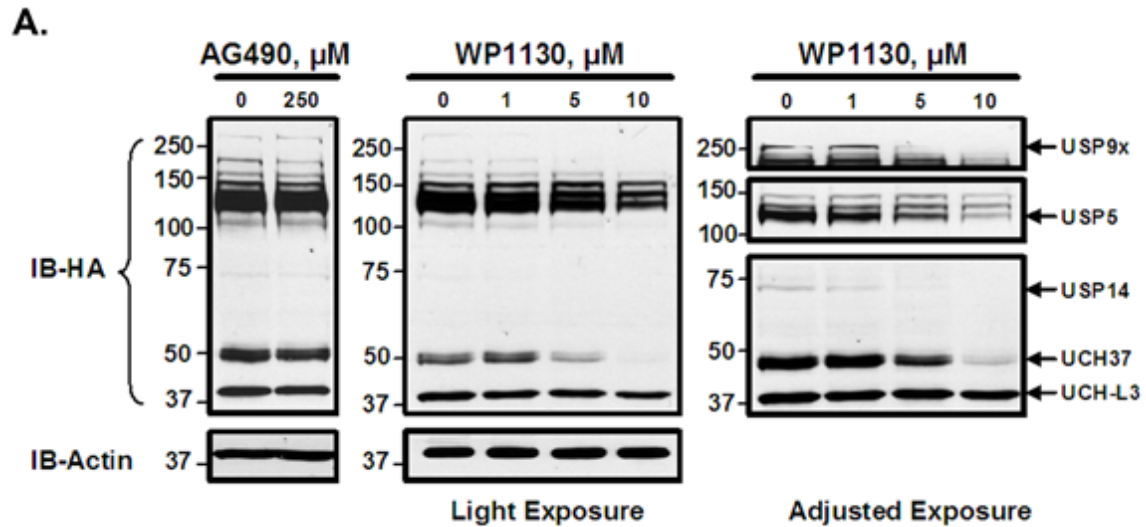


Figure 17: DUB inhibitory profile of WP1130.

(A). Z138 cells were treated with the indicated concentration of WP1130 or AG490 (250 μM) for 1 hours. The cells were lysed in DUB labeling buffer as described in the materials and methods. 20 μg of clarified supernatant was incubated with 200 nM of HA-UbVS for 1 hr at 37°C. HA immunoblotting was used to assess changes in DUB labeling. Actin was probed as a protein loading control. Dual exposures are shown to allow determination of DUB inhibition against DUBs with high and low labeling activity. Individual DUBs were assigned (right) based on relative electrophoretic mobility and confirmation by subsequent immunoblotting (not shown).

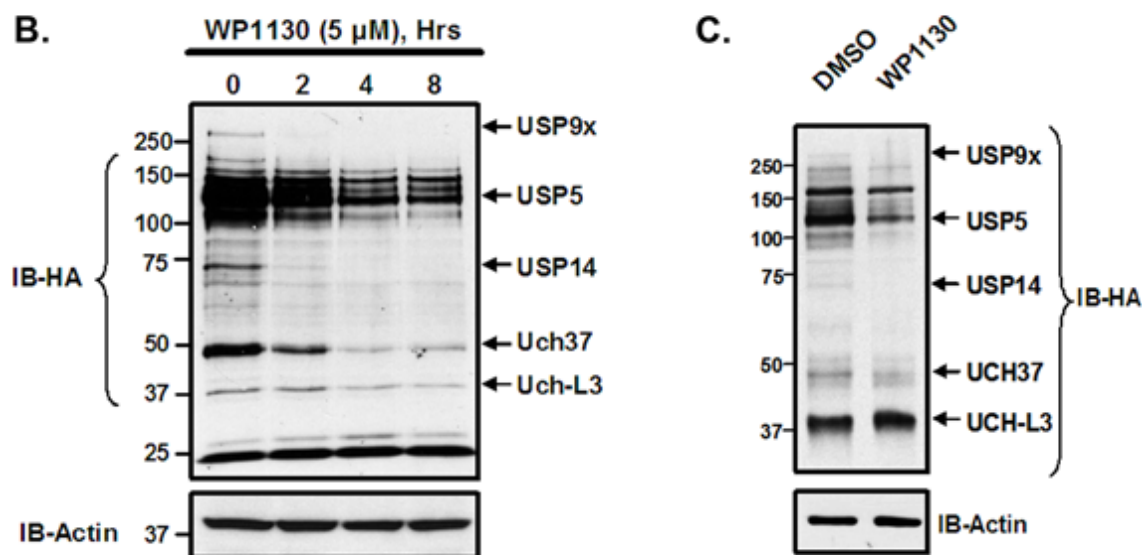


Figure 17: DUB inhibitory profile of WP1130 (continued).

(B). Z138 cells were treated with 5 μ M WP1130 for the interval indicated followed by lysis in DUB buffer. Lysates were subjected to HA labeling and DUB assignments were made based on the criterion described in A.

(C). Untreated Z138 cell extracts were prepared in DUB buffer as described in the materials and methods. 20 μ g of lysate were incubated with DMSO or 5 μ M WP1130 for 1 hr at 37°C. The reaction mixture was subjected to labeling with 200 nM HA-UbVS for an additional 1 hr at 37°C and DUB activity was assessed by HA immunoblotting.

C11. Direct Inhibition of DUBs by WP1130

To determine whether WP1130 directly inhibits DUB activity, we incubated purified DUBs such as USP5, UCH-L1 and UCH-L3 with DMSO and WP1130 for 30 min in DUB buffer at 37°C. USP9x was immunoprecipitated from Z138 cell lysates, prepared in DUB buffer and the beads were incubated with DMSO or WP1130 for 30 min. Ub-AMC (500 nM) was added to each reaction and fluorescence was monitored every minute for up to 30 minutes. The maximum fluorescence observed at the end of the linear phase of substrate cleavage in DMSO or WP1130 treated DUBs was used as a gauge to estimate DUB activity and % inhibition. As shown in Figure 18A, treatment with 5 μ M WP1130 reduced the activities of USP9x, USP5 and UCH-L1 by 60 % or more (detail in 18C). No inhibition was observed against UCH-L3 activity, suggesting that WP1130 may be partly selective. We confirmed the loss of USP5 activity using HA-UbVs labeling, which demonstrated ~80% reduction in HA labeling upon incubation with WP1130 (Figure 18B).

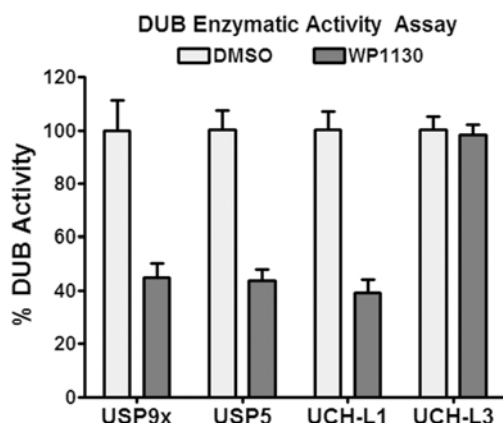
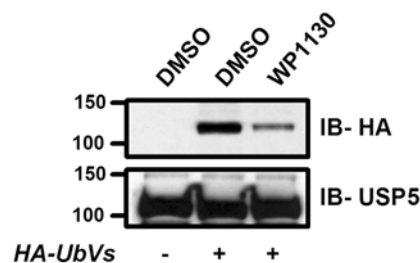
A.**B.**

Figure 18: WP1130 inhibits purified deubiquitinases.

(A). Optimal concentrations of recombinant or immuno-purified deubiquitinase enzyme (USP5; 20 nM, UCH-L1; 20 nM, UCH-L3; 5 nM, USP 9x; immuno-isolated) were incubated in DUB activity buffer containing WP1130 or DMSO (v/v) in a 100 μ l reaction volume for 30 min at 37°C in 96-well fluorometry plates. After incubation, 500 nM Ub-AMC was added to the reaction and the release of AMC-fluorescence was recorded over time. The % activity for each enzyme was estimated by monitoring the change in substrate cleavage relative to DMSO treatment (representing 100% activity).

(B.) Recombinant USP5 (20 nM) was subjected to HA-UbVs labeling in the presence of DMSO or 5 μ M WP1130 (30 min). Immunoblotting was used to determine USP5 activity (HA blotting - top) and protein level (USP5 blot –bottom).

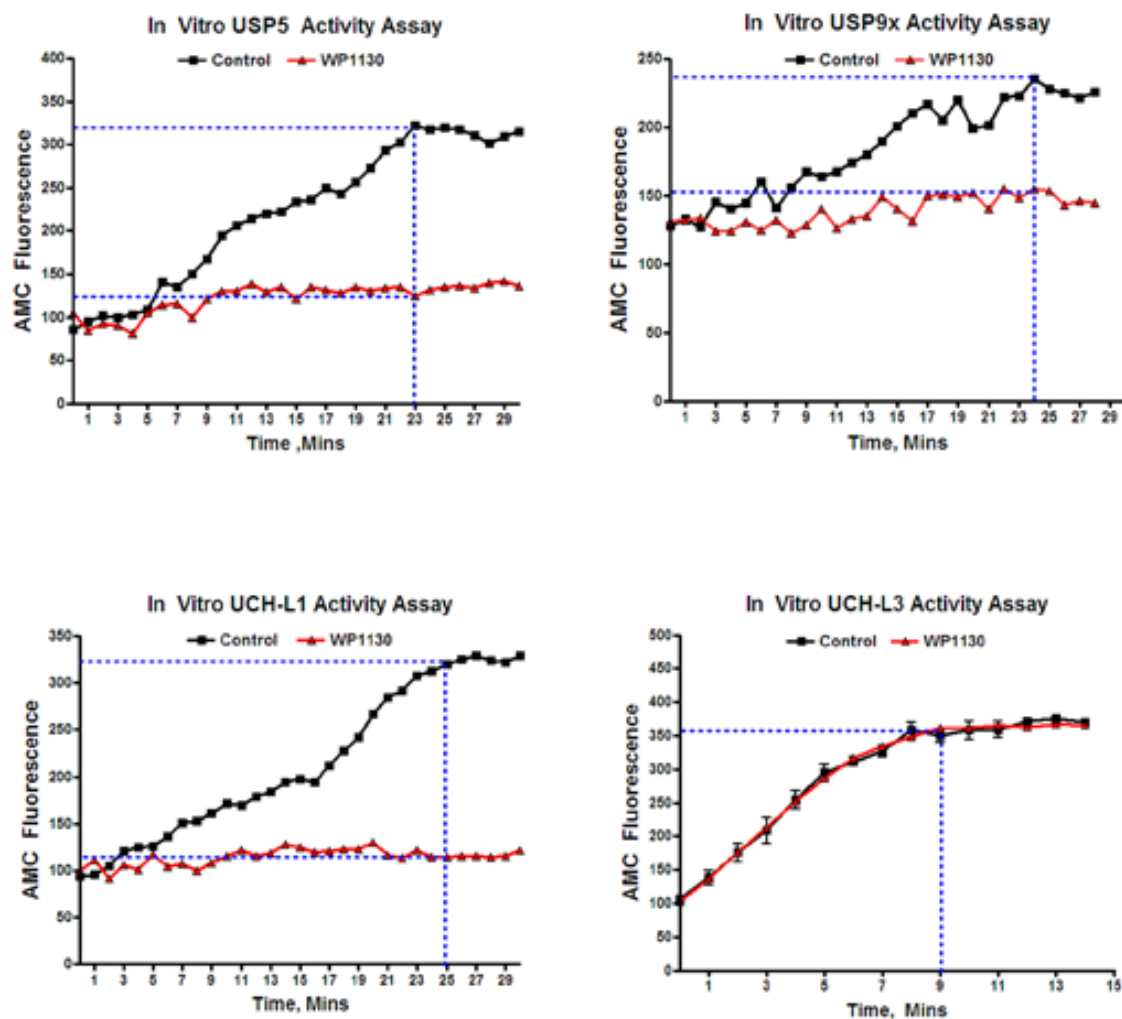


Figure 18: WP1130 inhibits purified deubiquitinases.

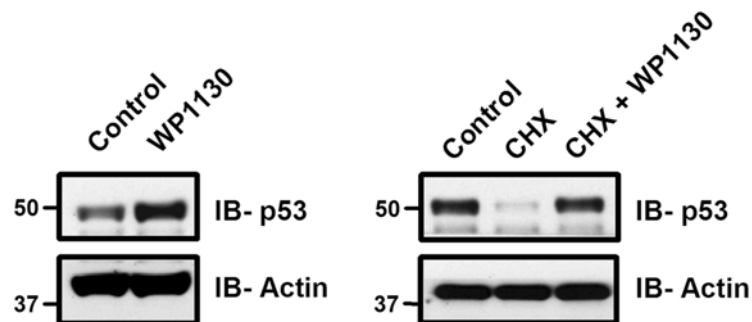
(C). Optimal concentrations of recombinant or immuno-purified deubiquitinase enzyme (USP5; 20 nM, UCH-L1; 20 nM, UCH-L3; 5 nM, USP 9x; immuno-isolated) were incubated in DUB activity buffer containing WP1130 or DMSO (v/v) in a 100 μ l reaction volume for 30 min at 37°C in 96-well fluorometry plates. After incubation, 500 nM Ub-AMC was added to the reaction and the release of AMC-fluorescence was recorded over time.

C12. WP1130 Modulates Pro- and Anti-Apoptotic proteins

USP5 is known to play a major role in maintaining the levels of unanchored polyubiquitin chains (181). Loss of USP5 has been reported to stabilize p53, due to the accumulation of free polyubiquitin chains which compete with ubiquitinated p53 (169). During the preparation of this dissertation, USP9x was described to enhance tumor cell survival by deubiquitinating the anti-apoptotic protein MCL-1, thereby promoting its stability (158). The authors showed a positive correlation between the increased levels of MCL-1 and USP9x in lymphomas and multiple myelomas. siRNA mediated knockdown of USP9x led to rapid degradation of MCL-1, and sensitization of tumor cells to apoptotic stimuli. Considering these recent reports, we investigated the downstream effects of inhibiting USP9x and USP5 in Z138 cells.

Consistent with published reports, we observed an increase in p53 protein levels upon WP1130 treatment (Figure 19A, left). Co-treatment of Z138 cells with cyclohexamide (50 μ g/ml) and WP1130 confirmed the stabilization rather than induction of p53 protein, as shown in Figure 19A (right). Furthermore, a rapid decline in MCL-1 levels upon WP1130 treatment was observed (Figure 19B). To re-confirm the role played by USP9x and USP5 in regulating the levels of MCL-1 and p53 respectively, we performed siRNA based knockdown of USP9x and USP5 in HEK293T cells. Loss of USP9x led to ~50% decline in MCL-1 levels while loss of USP5 showed ~2-fold increase in p53 levels (Figure 20). Together these results demonstrate that WP1130 inhibits selective cellular DUB activities, modulating the stability of both anti- and pro-apoptotic proteins.

A.



B.

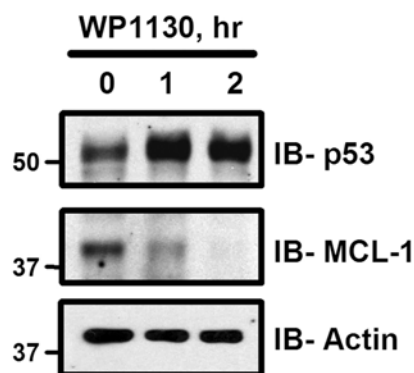


Figure 19: Effect of WP1130 on DUB regulated proteins.

(A). Left - Z138 cells were incubated with DMSO (Control) or 5 μ M WP1130 for 2 hours before total cell lysates were immunoblotted for p53 or actin as a protein loading control. **Right** – Z138 cells were treated with DMSO (Control), cycloheximide (CHX, 50 μ g/ml) alone or combined with 5 μ M WP1130 for 1 hr before whole cell lysate were immunoblotted for p53 and actin as a protein loading control.

(B). Whole cell extracts from Z138 cells were probed for the protein levels of MCL-1 and p53 after WP1130 (5 μ M) treatment for the indicated intervals. WP1130 treated cells show a rapid down-regulation of MCL-1 and accumulation of p53.

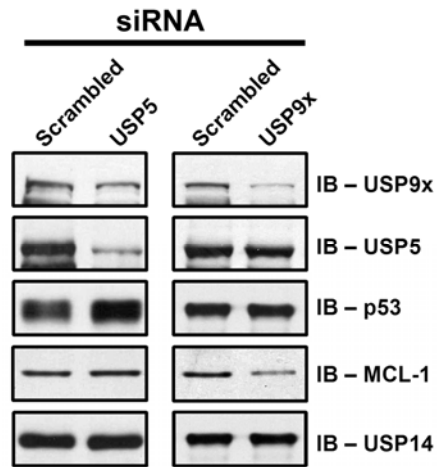


Figure 20: Knockdown of USP5 and USP9x regulates p53 and MCL-1 levels.

HEK293T cells were transfected with 30 nM siRNA targeting specific DUBs as described in the materials and methods. Extent of each DUB (USP9x, USP5) knockout and their impact on previously described targets was assessed by immunoblotting for p53, MCL-1 and USP14 (negative control, no impact).

C13. Inhibitory effects of WP1130 are Thiol-Sensitive

WP1130 contains α , β unsaturated ketone group which are highly reactive towards sulfhydryl containing reagents via Michael's addition reaction (182). Therefore, we investigated if the presence of reducing agents such as DTT has any impact on WP1130 mediated inhibition of the Jak2-Stat3 pathway and apoptosis. As shown in Figure 21A, the accumulation of Jak2 and ubiquitinated proteins in the insoluble fraction of WP1130 treated cells was completely blocked by the presence of DTT. Assessment of Jak2 ubiquitination from these cells showed a near complete inhibition of WP1130-mediated ubiquitination of Jak2 (Figure 21B). These results prompted us to investigate whether IL-6 induced Stat3 phosphorylation, which is inhibited by WP1130, can be rescued in the presence of DTT. We observed a complete restoration of IL-6 driven Jak2/Stat3 phosphorylation when Z138 cells were treated with combination of WP1130 and DTT (Figure 21C, Lane 5). The cytotoxic effects of WP1130 were also completely abolished in the presence of DTT (Figure 21D). Accordingly, no DUB inhibition was seen in WP1130 and DTT co-treated Z138 cells (Figure 21E). This observation also correlates with the loss of *in vitro* DUB inhibition by WP1130 in the presence of DTT (Figure 21F). Such interference in WP1130's growth-inhibitory or Jak2-inhibitory activities by DTT should not be confused with a possible oxidative stress induced by WP1130. To validate this, we examined the release of reactive-oxygen species (ROS) in Z138 cells treated with WP1130 and H₂O₂ (positive control) using DCFDA. No increase in the generation of any reactive-oxygen species was observed (Figure 22) in WP1130 treated cells, suggesting WP1130 does not induce an oxidative-stress which can be countered in the presence of DTT. Furthermore, oxidative stress has been suggested to activate, not inhibit, the Jak2/Stat3 pathway. AG490 itself has been shown to act as a reducing agent (183).

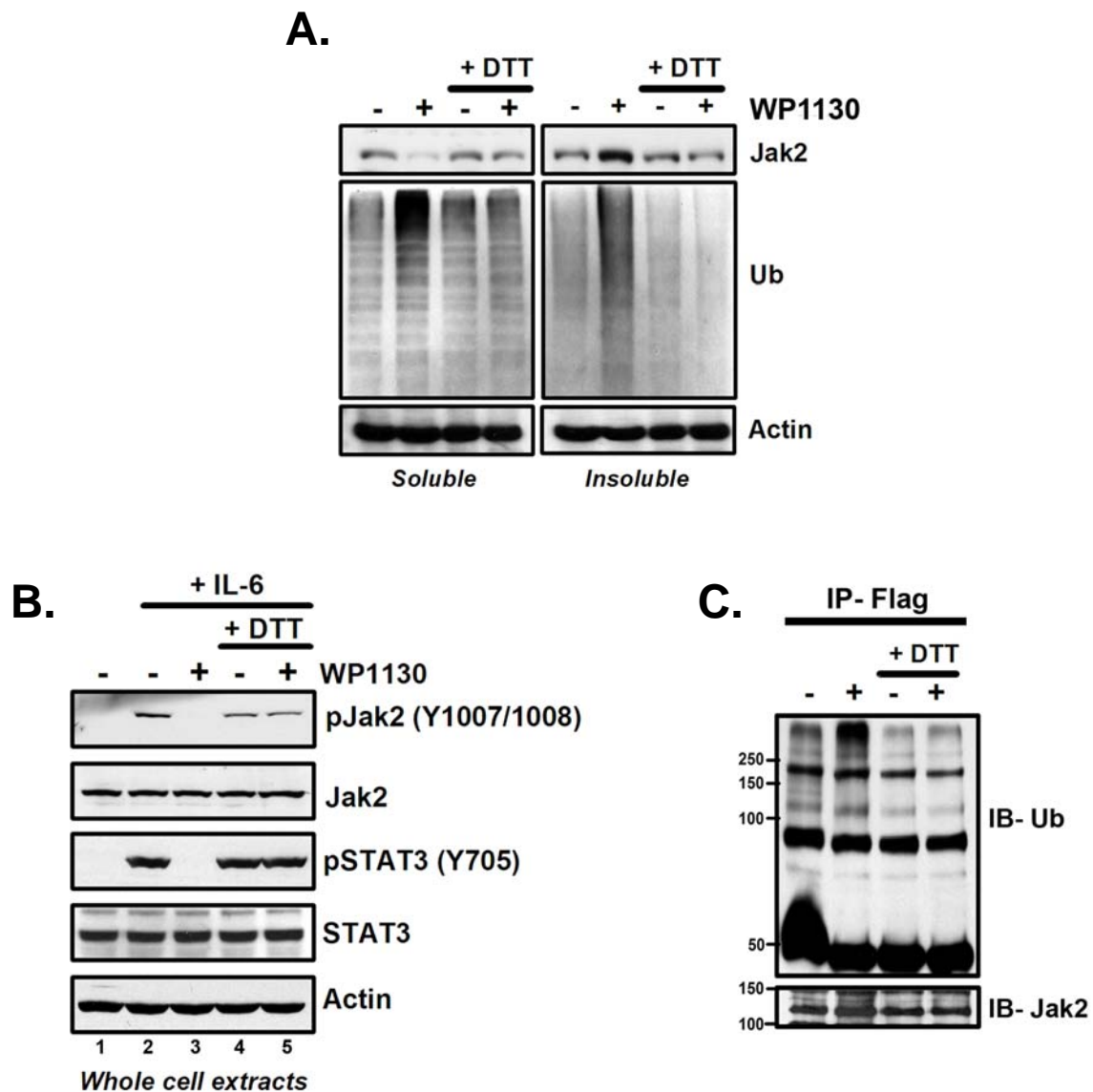


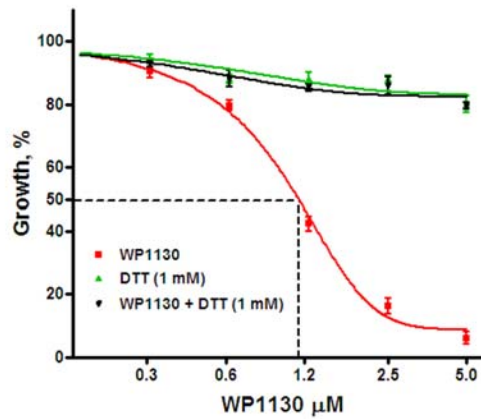
Figure 21: Inhibition of Jak2 signaling and protein ubiquitination by WP1130 is thiol-sensitive.

(A). Z138 cells were treated with DMSO (-), WP1130 (+) in the absence and presence of 1 mM DTT for 2 hours. The lysates were prepared and probed for the presence of ubiquitinated proteins as well as Jak2 in the detergent-soluble and insoluble fraction of WP1130 treated cells.

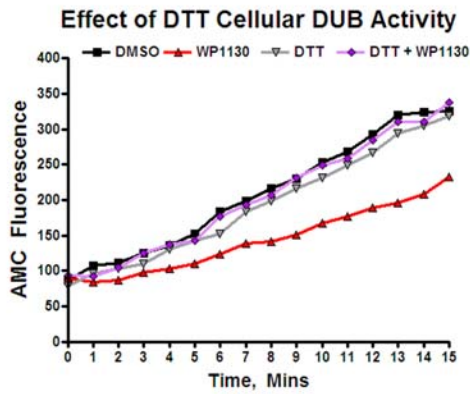
(B). Z138 cells were treated with DMSO (-), WP1130 (+) in the absence and presence of 1 mM DTT for 2 hours. Following treatment, the cells were stimulated with 10 ng/ml IL-6. The cells were lysed to prepare whole cell extracts and probed for the activation of the Jak2-Stat3 pathway.

(C). Flag-Jak2 HEK293 cells were treated with DMSO (-), WP1130 (+) in the absence and presence of 1 mM DTT for 2 hours. The cells were lysed in denaturing conditions to immunoprecipitate Jak2.

D.



E.



F.

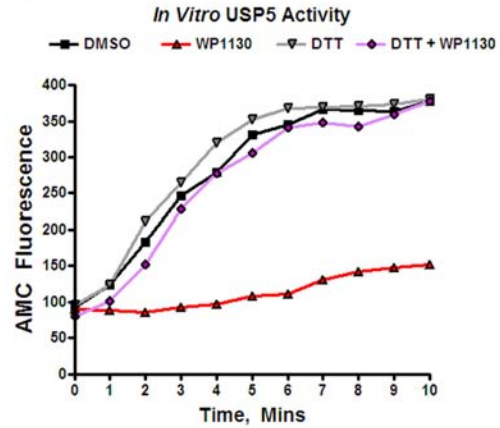


Figure 22: Inhibition of Jak2 signaling and protein ubiquitination by WP1130 is thiol-sensitive (continued).

(D). Z138 cells were incubated with increasing concentration of WP1130 in the presence and absence of 1 mM DTT for 3 days. The effect of WP1130, DTT alone or DTT+WP1130 on Z138 proliferation was assayed using MTT reagent.

(E). Lysates from DMSO, WP1130, DTT and DTT+WP1130 treated cells were incubated with 500 nM Ub-AMC to detect the changes in DUB activity. AMC fluorescence was monitored at 380/460 em/ex.

(F). Purified USP5 was incubated with DMSO, WP1130, DTT or DTT+WP1130 for 30 min. The DUB activity was monitored by incubating the substrate, Ub-AMC.

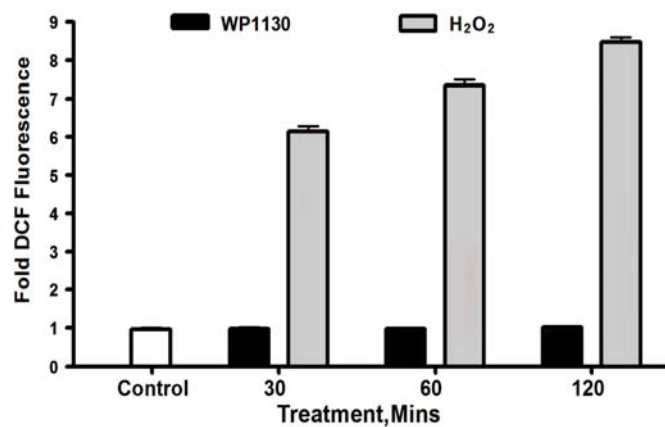


Figure 22: WP130 does not induce reactive-oxygen species.

Z138 cells were incubated with WP1130 (5 μ M, black bars). DMSO (white bar) and H₂O₂ (1 mM, gray bars) for 1 hour. The cells were then washed twice in PBS, and incubated with 10 μ M H₂-DCFDA. After 10 mins of incubation, the cells were collected and the fluorescence of DCFDA was recorded as an indicator of generation of reactive-oxygen species.

D. Discussions

D1. Suppression of Jak-Stat Signaling by WP1130

Given the essential role for Jak-Stat signaling in growth-promoting activities via a variety of cytokines, constitutive activation of Jaks would be expected to have pathological consequences. The Jak-Stat pathway is often found to be activated in most forms of cancer [Reviewed in (44), (45)]. Several growth factors and cytokines are expressed in most forms of cancer, including solid tumors and hematological malignancies, leading to autocrine activation of the Jak-Stat pathway inducing proliferative and anti-apoptotic effects (184-186). In addition, chromosomal translocations as well as point mutations within Jak2 have been shown to constitutively activate Jak2 kinase, independent of cytokine signaling. Many Jak2-fusion proteins/mutations have been implicated in many hematological malignancies. Taken together, these reports project Jak2 as an attractive therapeutic target in cancer, validating the development of many small molecule inhibitors against Jak2 (187, 188).

Before the spur in the development of Jak2 inhibitors, tyrphostin AG490 was the only described inhibitor of Jak2 which induced tumor cell apoptosis and displayed anti-tumor activities (69). Although AG490 inhibited the kinase activity of Jak2, requirements for high μM concentration limited its clinical potential and development. To develop a more potent inhibitor of Jak2 from the AG490 chemo-type, our research group screened a small library of AG490 analogues in cell based assays. WP1130, a lead compound from such a screen, inhibited cytokine-dependent (IL-3, IL-6) as well as constitutively active Jak2-Stat3 signaling at low μM concentrations. Although WP1130 is an analogue of AG490, it is unique and distinct from parental tyrphostin in its mechanism of action. While AG490 is a well known inhibitor of Jak2 kinase activity, WP1130 demonstrated no inhibition of Jak2-kinase activity (Figure 1 and 2).

Initial studies with previous derivatives of WP1130 (WP1066) showed that WP1066 promotes Jak2 degradation (77), which leads to the suppression of downstream signaling. We too had observed a similar loss of Jak2 protein from the soluble lysates (Figure 4). However, we could not prevent the WP1130 mediated down-regulation of Jak2 in the presence of major protease inhibitors (Figure 6). To elucidate the mechanism of suppression of Jak2-Stat3 signaling by WP1130, we modified our lysate preparation protocol to investigate the detergent-insoluble lysate as well. We observed a rapid, time- and concentration-dependent appearance of Jak2 in the detergent-insoluble fraction of WP1130 treated cells (Figure 7). Such a spatial relocalization of Jak2 from its normal cellular localization (cytosol, membrane) into the detergent-insoluble fraction prevents Jak2 from activating the downstream effector Stat proteins. Furthermore, using cellular fractionation studies as well as microscopic examination, our data provides several lines of evidence which show that WP1130 promotes the accumulation of Jak2 into signaling incompetent aggresomes (Figure 10-13). WP1130-induced sequestration of Jak2 from soluble to the insoluble fraction was highly specific for Jak2, as other members of Jak family (Jak1, Tyk2) did not show similar levels of accumulation in the insoluble fraction (Figure 7).

The accumulation of Jak2 into the detergent insoluble fraction of WP1130 treated cells was accompanied by its rapid polyubiquitination. Immunoprecipitation of Jak2 under denaturing conditions from Z138 (endogenous) or from Flag-Jak2 overexpressing HEK293 cells showed ubiquitination of Jak2 (Figure 14). Previous reports have described SOCS-1 dependent polyubiquitination of Jak2 in response to cytokine stimulation, which leads to its proteasomal degradation (38, 189). We observed an increased association between Jak2 and SOCS-1 after WP1130 treatment, which explains the rapid ubiquitination of Jak2. However, the ubiquitination of Jak2 in WP1130 treated cells was independent of any cytokine-stimulation or new

protein synthesis, suggesting that WP1130 amplifies the resident, endogenous Jak2 ubiquitination pathway many-fold, leading to its hyper-ubiquitination.

Using overexpression of lysine-specific mutants of HA-tagged ubiquitin, we observed a major accumulation of lysine-63 (K-63) linked ubiquitinated of Jak2 in WP1130 treated cells (Figure 15). On the other hand, we did not observe any significant increase in the accumulation of lysine-48 (K-48) linked polyubiquitinated Jak2 in WP1130 treated cells. Previous studies have demonstrated proteasomal degradation of ubiquitinated Jak2 (38). However, the proteasomal degradation of a protein is often attributed to the formation of lysine-48 linked polyubiquitin chains (86). The ubiquitination of Jak2 using K-63 linked ubiquitin chains is novel and has not previously been described. This is also in accordance with previous studies which have reported the accumulation of K-63 linked proteins into the insoluble fraction and aggresomes (175, 190).

We further showed that the inhibition of Jak2-Stat3 signaling pathway is linked intricately to the ubiquitination of Jak2. Since WP1130 contains an α , β -unsaturated carbonyl group, it can interact with sulfhydryls through a Michael addition reaction (182). Such a modification should interfere with the anti-tumor activities of WP1130, and the suppression of Jak2-Stat3 signaling. We observed a complete restoration of Jak2-driven Stat3 activation in cells treated with WP1130 and DTT. The accumulation of Jak2 into the detergent-insoluble fraction was also completely abolished with the co-treatment of WP1130 and DTT. This was further in correlation with the absence of Jak2 ubiquitination in those cells (Figure 21). It should be noted that the DTT-induced interference in WP1130's activities was not attributed to induction of oxidative stress (by WP1130) that could be quenched by a reducing agent (DTT). We had observed no change in reactive-oxygen species (ROS) in WP1130 treated cells, which ruled out induction of oxidative stress as a contributor to its activity. Furthermore, oxidative stress

has been rather shown to *activate* the Jak2-Stat3 signaling pathway, not inhibit it (191) (Figure 22).

In summary, our study shows that WP1130 potently inhibits the Jak2-Stat3 pathway by a unique mechanism involving rapid ubiquitination and aggresomal deposition of Jak2. In doing so, WP1130 blocked the activation of downstream Stat3 signaling, contributing to the induction of apoptotic cell death.

D2. Modulation of DUB activity by WP1130

Ubiquitination and ubiquitin-like protein modification plays a major role in directing the fate and function of most cellular proteins (78). Several key enzymes in these pathways are amplified, or modified in diseased cells and provides rationale for development of small molecules that inhibit or modulate their activity. Major advances have been made in selective targeting of specific enzymes in these pathways; some with clinical impact (192). Due to the specialized role of DUBs in the ubiquitin cycle and their emerging role in control of multiple signaling pathways and oncoproteins, DUB inhibitors may be useful anti-cancer agents (164). In this manuscript, we describe the unexpected DUB inhibitory activity of WP1130; a novel small molecule derived from the Jak2 inhibitor AG490. WP1130 induces accumulation of polyubiquitinated proteins and promotes tumor cell apoptosis.

Peptide based potent, irreversible inhibitors of DUBs, such as ubiquitin aldehyde and ubiquitin vinyl sulfone (UbVS) have been previously described (165, 166). However, their therapeutic potential is limited by their high mol wt and limited cellular bioavailability. Other small molecule compounds, such as D12-prostaglandin J2 were initially shown to inhibit ubiquitin iso-peptidase activity in cells (IC_{50} 30 μ M) and cause cellular accumulation of ubiquitinated proteins and cell death (176). A key molecular determinant required for DUB inhibitory activity, α , β -unsaturated ketone with sterically accessible β -carbon, was noted in this compound and lead to the identification of additional inhibitors with similar activities, such as dibenzylideneacetone (DBA; IC_{50} 20–40 μ M), curcumin (IC_{50} 80–100 μ M) and shikoccin (NSC-302979; IC_{50} 15 μ M). However, the profile of specific DUBs affected by these compounds has not been described (167).

Using multiple *in vivo* and *in vitro* assays, we provide mechanistic evidence that WP1130 acts as a partially selective DUB inhibitor. Our data shows that WP1130 suppresses the activities of major cellular deubiquitinases such as USP5, UCH-L1, USP9x, USP14 and UCH37. Inhibition of multiple DUBs is likely to induce multiple and predictable cellular changes, such as: (i) increased accumulation of polyubiquitinated proteins/unanchored polyubiquitin chains (Figure 16), (ii) decline in the pool of monomeric ubiquitin (Figure 16A), (iii) slower rate of polyubiquitin disassembly (Figure 16C), (iv) an overall decrease in individual DUB activities (Figure 18) and (v) effect cellular levels/activities of DUB regulated oncoproteins (Figure 19).

A marked increase in cellular ubiquitinated proteins, arising either due to proteasome inhibition or loss of cellular DUB activity can trigger aggresome formation (174, 177). Formation of aggresomes under conditions of stress results in a temporary cellular cytoprotective event, relocating vast amounts of accumulated ubiquitinated proteins to the aggresomal insoluble fraction (193). Our recent data suggests that WP1130 treatment causes accumulation of polyubiquitinated Jak2 and Bcr-Abl into detergent insoluble aggresomes, thereby suppressing tumor cell proliferation (Kapuria et al, Sun et al, unpublished data). Therefore, trafficking of oncoproteins which play a crucial role in proliferation, survival and growth factor signaling into the aggresome, where they are unable to function, is predicted to be detrimental to tumor cells. However, the DUB(s) that play a crucial role in regulating these oncogenic kinases are not currently known. Using xenograft mouse models of CML and melanoma, we have previously shown that WP1130 treatment effectively suppresses tumor growth *in vivo* (73). Together these observations suggest that WP1130 may act as a therapeutic agent through its effects on DUB activity.

It is noteworthy that several of the DUBs targeted by WP1130 have recently been shown to be key regulators of the stability and turnover of specific oncogenes and

apoptotic regulators, including Mcl-1 (158) and p53 (169). Other DUBs are suggested to play a direct role in transformation (153) and control of ubiquitinated protein entry into the proteasome (194). Still others that may be targeted by WP1130 have recently been shown to play a role in unregulated cell growth and tumor cell addiction (159). The role played by many other DUBs and their impact on their cognate substrates in the apoptotic activity engaged by WP1130 is not yet known.

The molecular basis of DUB inhibition by WP1130 is currently unknown. WP1130 contains α , β -unsaturated carbonyl group that can hypothetically interact with sulfhydryl of cysteines found in the active sites of DUBs through a Michael addition reaction (182). However, similar carbonyl groups are found in AG490 but this molecule possesses no apparent DUB inhibitory activity in cells. Whether WP1130 inactivates DUBs by interacting with their active site cysteines remains to be determined. If so, other thiol-containing reagents such as DTT may also react with WP1130 and quench its activities. Interestingly, we have observed near complete loss of WP1130 mediated ubiquitination and apoptosis in the presence of DTT. This observation correlates with the loss of DUB inhibition by WP1130 in the presence of DTT (Figure 21). These observations may also suggest that active site cysteines and structural changes induced by sulfhydryl reduction and oxidation of DUBs may play a role in WP1130-mediated DUB inhibitory activity and selectivity as well.

The full profile of DUBs that are affected by WP1130 is being further assessed. Crystal structures of several deubiquitinating enzymes have revealed a high degree of homology within their catalytic core (195, 196). This may account for the partial selectivity of WP1130 against a specific subset of DUBs. Further SAR studies to develop structural variants of WP1130 with increased target specificity may enhance anti-tumor directed activities.

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E. MANUSCRIPTS PARTIALLY OR FULLY RELATED TO THIS DISSERTATION

1. Geoffrey A. Bartholomeusz, Moshe Talpaz, **Vaibhav Kapuria**, Ling Yuan Kong, Shimei Wang, Zeev Estrov, Waldemar Priebe, Ji Wu, and Nicholas J. Donato. 2007. Activation of a novel Bcr/Abl destruction pathway by WP1130 induces apoptosis of chronic myelogenous leukemia cells. Blood 109:3470-3478.
2. **Vaibhav Kapuria**, Luke F Peterson, William G Bornmann, Moshe Talpaz, Nicholas Donato. 2010. Small molecule mediated deubiquitinase inhibition results in aggresome formation and tumor cell apoptosis. Submitted to Blood.
3. Hanshi Sun [‡], **Vaibhav Kapuria** [‡], Dexing Fang, Geoffrey Bartholomeusz, Luke F Peterson, William G Bornmann, Moshe Talpaz, Nicholas Donato. 2010. Inhibition of Bcr-Abl and USP9x leads to apoptosis in CML cells. Submitted to Blood.

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