Article Title: Targeting deubiquitinase activity with a novel small molecule inhibitor as therapy for B-cell malignancies

Short title: Usp9x/Usp24 as therapeutic targets in B-cell tumors

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Key Points

- Deubiquitinases Usp9x and Usp24 regulate Mcl-1 and myeloma cell survival.
- Small molecule-mediated Usp9x/Usp24 inhibition induces apoptosis and blocks myeloma tumor growth in vivo.

Abstract

Usp9x was recently shown to be highly expressed in myeloma patients with short progression-free survival and is proposed to enhance stability of the survival protein, Mcl-1. In the present study we found that the partially selective Usp9x deubiquitinase inhibitor WP1130 induced apoptosis and reduced Mcl-1 protein levels. However, shRNA mediated knockdown (KD) of Usp9x in myeloma cells resulted in transient induction of apoptosis, followed by a sustained reduction in cell growth. A compensatory up-regulation of Usp24, a deubiquitinase closely related to Usp9x, in Usp9x knockdown (KD) cells was noted. Direct Usp24 KD resulted in marked induction of myeloma cell death that was associated with a reduction of Mcl-1. Usp24 was found to sustain myeloma cell survival and Mcl-1 regulation in the absence of Usp9x. Both Usp9x and Usp24 were expressed and activated in primary myeloma cells while Usp24 protein overexpression was noted in some drug-refractory myeloma patients and other B-cell malignancies. Further, we improved the drug-like properties of WP130 and demonstrate that the novel compound EOAI3402143 dose-dependently inhibited Usp9x and Usp24 activity, increased tumor cell apoptosis and fully blocked or regressed myeloma tumors in mice. We conclude that small molecule Usp9x/Usp24 inhibitors may have therapeutic activity in myeloma.

Introduction

The success of bortezomib and lenalidomide in treatment of some B-cell tumors highlights the potential for additional agents that target components of the ubiquitin/proteasome cycle to have therapeutic value. Recent studies provide evidence for the overexpression of specific deubiquitinases (DUBs) in cancer, including some specific to multiple myeloma (MM) and possibly other B-cell malignancies. Usp7 was recently shown to be overexpressed in MM patients, where it regulates ubiquitination and turnover of p53 and other proteins. One recently described Usp7 inhibitor has demonstrated anti-MM activity in pre-clinical animal models. Other inhibitors targeting two DUBs that are components of the 19S proteasome have been shown to have activity against several tumors, including myeloma. Usp9x has received considerable attention as a potential therapeutic target for several cancers since it has been reported to regulate ubiquitination and half-life of Mcl-1, a key anti-apoptotic protein detected in...
many tumors. Usp9x was also recently shown to suppress chemo-, radio- and targeted-therapy in several tumor types through its regulation of Mcl-1 and other proteins. Mcl-1 is essential for tumor cell survival and high levels are noted in drug resistant MM patients. Using proteomics, ubiquitinated Mcl-1 was recently shown to be bound and deubiquitinated by Usp9x, thus preventing its destruction by the proteasome. Usp9x expression was shown to be highly elevated in MM patients and correlated with poor patient prognosis. In other B-cell malignancies Usp9x levels were associated with elevated Mcl-1 protein content. Therefore, inhibitors of Usp9x activity may function as a form of “targeted” therapy for B-cell tumor patients.

Deubiquitinase (DUB) inhibitor WP1130, previously known as Degrasyn, is a partially selective DUB inhibitor shown to inhibit deubiquitinating activity of Usp9x, Usp5, Usp14 and UCH37. Usp9x inhibition by WP1130 has been shown to promote apoptosis by reducing Mcl-1 levels and increasing tumor cell sensitivity to chemotherapy. Also, WP1130-mediated Usp9x inhibition blocks the growth of ERG-positive prostate tumors in vitro and in mouse xenograft models of prostate cancer.

To understand the role of Usp9x overexpression in MM and other B-cell tumors, we interrogated the impact of its silencing and small molecule-mediated inhibition. We found that knockdown of Usp9x in MM cells led to an early onset of apoptosis, followed by sustained growth suppression by blocking G2/M cell cycle transition. Unexpectedly, we also noted that Usp9x knockdown (KD) led to a compensatory activation and up-regulation of a closely-related DUB, Usp24, which shows a great degree of biochemical and sequence similarity to Usp9x. We found that Usp24 also plays a role in myeloma cell survival through its regulation of Mcl-1 levels, particularly in the absence of Usp9x. We finally assessed the anti-myeloma activity of a small molecule DUB inhibitor derived from WP1130 with more specific activity against Usp9x and Usp24. The results suggest that DUB inhibition presents an additional therapeutic approach in the treatment of myeloma and other B-cell tumors.

Methods
Compounds: WP1130 and EOAI3402143 (G9) were synthesized by methods outlined in patent applications. Bortezomib was provided by Millennium Pharmaceuticals. MG132 was purchased from Cayman Chemical Co. All reagents were made up and stored frozen as 10 mM stock solutions.

Measurement of DUB: DUB activity in cell lines and patient samples was measured as previously described.

Lysate preparation and Western blotting were performed as previously described.
Other methods used in this study can be found in the supplement.

**Results**

**WP1130 inhibits Usp9x activity and promotes Mcl-1 destruction in B-cell tumors**

Previously we described a small molecule DUB inhibitor, WP1130, with activity against Usp9x. To determine whether WP1130-mediated Usp9x inhibition induced a corresponding change in Mcl-1 protein levels in myeloma and lymphoma, 4 established myeloma cell lines (MM1.R, H929, MM1.S and RPMI 8226) and 2 mantle cell lymphoma (MCL) cell lines (Z138 and Mino), were treated with WP1130 before assessing Usp9x activity and Mcl-1 protein levels. As shown in figure 1A and B, WP1130 induced time-dependent inhibition of Usp9x activity, effective reduction in Mcl-1 protein levels and induction of apoptosis detected by PARP cleavage. Both WP1130 and proteasome inhibitors (MG132 and bortezomib) increased Ub protein content (Fig. 1C); however, WP1130-mediated Mcl-1 reduction was blocked by pretreatment with proteasome inhibitors (MG132 and bortezomib), confirming a role for ubiquitin-regulated/proteasomal-dependent destruction of Mcl-1 in WP1130 treated cells.

To determine whether Usp9x activity and Mcl-1 levels were measurable and sensitive to inhibition by WP1130 in primary tumor cells, freshly isolated CD138 positive cells from newly diagnosed or drug-refractory myeloma patients (Fig. 2A and B) (characteristics tabulated in Table 1) or highly purified chronic lymphocytic leukemia (CLL) cells from patients (Fig. 2C) were treated with WP1130 for 4 hours before assessing total DUB activity. WP1130 displayed partially selective Usp9x inhibitory activity and was associated with reduced expression of Mcl-1 protein (Fig. 2A). WP1130 dose-dependent DUB inhibition was also assessed in patient samples (MM5500 and CLL60) where sufficient sample permitted and confirmed a close association between Usp9x inhibition and Mcl-1 destruction (Fig. 2B and 2C). DUB inhibition by WP1130 was also associated with a reduction in myeloma and plasmacytoma cell survival as assessed by the % CD138+/Annexin V- cell population (Fig. 2A and B).

**Effect of Usp9x knockdown on myeloma cell survival**

To assess the role of Usp9x in myeloma, shRNA retroviral vectors targeting control (LMP) or Usp9x sequences (HS1, HS2) were expressed in MM1.S cells and effects on Usp9x levels and cell survival were examined. Both HS1 and HS2 were effective in reducing Usp9x levels and MM1.S cell survival when compared to LMP expressing cells (Supplemental Fig. S1A). In order to examine if the Usp9x knockdown triggered a reduction in cell survival mediated through Mcl-1, subsequent studies compared cell survival in control, Usp9x knockdown (KD) and Mcl-1 KD MM1.S and H929 cells. As shown in figure 3A, Usp9x KD
H929 cells show a slight reduction in Mcl-1 basal levels but no effect was observed in Usp9x KD MM1.S cells. Usp9x KD in both H929 and MM1.S cells was not as effective as direct Mcl-1 KD in increasing apoptosis as detected by Annexin positivity (Fig. 3A-right). We also noted that viable cells emerged from the Usp9x knockdown that had reduced proliferative activity (Fig. 3B-top), primarily due to a block in exit from G2/M (Fig. 3B-bottom). After 4 to 5 division cycles, Usp9x KD cells with low level Usp9x protein expression remained 90 to 95% viable, but grew at a reduced rate when compared to control KD cells. We assessed basal and WP1130-sensitive DUB activity in control KD and chronic Usp9x KD H929 cells and determined that although Usp9x protein levels were reduced by >90%, DUB activity associated with Usp9x (Fig. 3C, top band, compare lanes 1 and 3) could not be distinguished between control KD and Usp9x KD cells. WP1130 treatment resulted in complete inhibition of high MW DUB activity and reduced Mcl-1 protein expression in both control KD and Usp9x KD cells. These results suggested that complete ablation of Usp9x activity was necessary to suppress myeloma cell survival and reduce Mcl-1 protein levels. These results were also consistent with the possibility of expression of a WP1130-sensitive DUB with Usp9x-like molecular characteristics that compensates for Usp9x deficiency. Usp24 bears a high degree of biochemical and sequence similarity to Usp9x. Lysates from H929 and MM1S chronic Usp9x KD cells showed increased Usp24 protein expression (Fig. 3D) when compared to control KD cells. To determine whether these changes were an artifact of Usp9x shRNA-mediated knockdown, we assessed DUB activity and Usp24 expression in two colon cancer cell lines with full genetic disruption of the Usp9x gene. HCT116<sup>Usp9x<sup>−/−</sup></sup> and DLD-1<sup>Usp9x<sup>−/−</sup></sup> cells had no detectable Usp9x expression but retained high molecular weight DUB activity that was associated with increased Usp24 protein expression (Fig. 3E), suggesting that Usp24 compensates for loss of Usp9x in multiple cell types. To confirm Usp9x and Usp24 expression and activity in myeloma cells, each DUB was subjected to immunodepletion prior to assessing DUB activity in H929 and MM1.S myeloma cells. As shown in figure 3F, immunodepletion of either DUB resulted in a partial reduction of high MW DUB activity (HA-Usp9x/Usp24) in both H929 and MM1.S cells, suggesting that both DUBs are active in myeloma cells. To assess potential clinical relevance, 11 primary myeloma samples from patient tumors with high or good risk cytogenetics were evaluated for Usp9x and Usp24 gene expression by qPCR and compared to control KD or Usp9x KD H929 cells (Fig. 3G). Usp9x expression was elevated (2 – 100 fold) compared to the H929 myeloma cell line, and was independent of the cytogenetic profile. Similarly, Usp24 expression was above H929 cellular levels in 8 of 11 samples, with all 8 co-expressing Usp9x and Usp24. Oncomine™ (Compendia Bioscience, Ann Arbor, MI) data acquired from Zhan et al. showed that the expression of both Usp9x and Usp24 was increased in MGUS (n=44) and myeloma (n=12) bone marrow when compared to normal bone.
marrow (n=22) (Supplemental Fig. S1C). Usp9x and Usp24 protein expression was compared in 2 newly diagnosed, 2 relapsed myeloma patients, 2 normal tonsilar B-cell preparations and representative (H929 and Z138) cell lines (Fig.3H). All 4 myeloma samples had Usp9x protein expression levels that were greater than those detected in tonsilar cells and established cell lines. Elevated Usp24 expression was detected in Z138 cells, newly diagnosed and drug refractory patient samples. Direct analysis of DUB activity in tonsilar CD138+ plasma cells and CD138+ myeloma and plasma cell leukemia showed increased Usp9x/Usp24 activity in tumor vs. normal plasma cells (Supplemental Fig. S1D). Usp24 protein was below detection in normal plasma cells and one myeloma sample. To determine whether other B-cell tumors also express these proteins, tissue microarrays assembled from patients with mantle cell lymphoma (MCL) and CLL were stained for either DUB and compared to various normal tissues. In MCL, 6 of 14 and 7 of 14, were positive for elevated Usp9x or Usp24, respectively, when compared to spleen or tonsil staining (Supplemental Fig. S2A). Three samples were positive for both DUBs. In CLL, 4 of 14 and 3 of 14 stained positive for elevated Usp9x and Usp24 expression, respectively, with 2 of 14 demonstrating dual positivity (Supplemental Fig. S2B). Examples of high and intermediate Usp9x and Usp24 positivity in MCL and CLL tumors are shown in figure 3I. These results suggest that B-cell tumors commonly overexpress one or both of these DUBs.

**Usp24 is essential for myeloma cell survival**

To assess the role of Usp24 in myeloma, two shRNA constructs were used to execute Usp24 knockdown in H929 and MM1.S myeloma cell lines (note both 24-1 and 24-2 were effective, Supplemental Fig. S1A). As shown in figure 4A, Usp24 levels were reduced by shRNA in either cell line and were associated with reduction in Mcl-1 protein expression. Further, Usp24 KD reduced cell survival in both H929 and MM1.S cells to a level greater than that achieved by direct Mcl-1 knockdown (Fig. 4B), suggesting that Usp24 plays a role in Mcl-1 stability and myeloma cell survival.

To confirm a complementary role of Usp24 and Usp9x in myeloma cell survival, siRNA (SmartPool) was used to knockdown either Usp9x or Usp24 in RPMI-8226 cells and the effect on Mcl-1 protein levels and cell survival was examined. Both Usp9x and Usp24 siRNA reduced Mcl-1 protein expression in RPMI-8226 cells (Fig. 4C). Further, cell survival was reduced by KD of either DUB, with greater levels of annexin staining consistently noted in Usp24 silenced cells (Fig. 4D). These results suggest that Usp24 plays a prominent role in myeloma cell survival and Mcl-1 regulation and increased Usp24 expression may compensate cell survival in the absence of Usp9x. Increased ubiquitination of immunoprecipitated Mcl-1
was observed in Usp24 KD cell lysate when compared to control lysates, consistent with a role for Usp24 in Mcl-1 deubiquitination (Supplemental Fig. S1E).

**Usp9x and Usp24 interact with Mcl-1**

Previous studies have demonstrated that Usp9x complexes with the N-terminus of Mcl-1. Since we observed that Usp24 upregulation modulated Mcl-1 protein expression levels in Usp9x silenced cells (Fig. 3), we wanted to examine if Usp24 also binds Mcl-1 and determine if this binding is constrained by the presence of Usp9x. Immunoprecipitation assays confirmed the association of endogenous Mcl-1 with Usp9x and Usp24 as demonstrated by the recovery of Mcl-1 protein after direct Usp9x or Usp24 pulldown and reciprocal recovery of either DUB by immunoprecipitation of Mcl-1 (Fig. 5A). To define determinants on Usp9x essential for Mcl-1 binding, full-length and deletion constructs of Flag-Usp9x (Fig. 5B) were co-expressed with Mcl-1 in HEK293T cells and subjected to Flag-pulldown, followed by blotting for Mcl-1. Full-length Usp9x with catalytic activity demonstrated weak but detectable Mcl-1 binding, which was absent in cells expressing a catalytically-inactive (CDM) mutant Usp9x protein (Fig. 5C). Partial deletion of the catalytic domain and the entire C-terminus of Usp9x (Flag-E1-USP9X) resulted in increased recovery of Mcl-1 while expression of just the N-terminal domain of Usp9x (Flag-E5-USP9X) did not retain Mcl-1 binding (Fig. 5C – right lane). Since the active site Cys (C1566) was retained in the E1 construct, we compared Mcl-1 binding to an E1 construct expressing Cys or Ala at position 1566 and did not detect an impact on Mcl-1 association (Fig. 5D). These results suggest that Mcl-1 binding is mediated through primary interaction with the central core of Usp9x and can be attenuated by other domains or catalytic activity.

Since Usp9x and Usp24 are closely related, we constructed a Usp24 expression vector that retained a portion of its central core and the entire C-terminus of Usp24 (HA-ΔN-Usp24) and assessed its Mcl-1 interaction in the presence or absence of full-length Flag-Usp9x (Fig. 5B). As shown in figure 5E, HA-ΔN-Usp24 retained Mcl-1 binding activity. In addition, co-expression with Usp9x led to recovery of Usp9x in the Usp24/Mcl-1 complex. Together, these results suggest that Usp9x and Usp24 are capable of Mcl-1 binding.

**Small molecule-mediated Usp9x/Usp24 inhibition**

WP1130 was the first small molecule described with Usp9x inhibitory activity and has shown activity against specific tumors *in vitro* and *in vivo*. However other DUBs are also WP1130 targets, with some recognized benefits and risks associated with the use of a partially-selective DUB inhibitor in a
clinical setting \(^4\). The main limitation for WP1130 as a clinical candidate is its low aqueous solubility (\(~2.3 \mu M\)) which limits its delivery and bioavailability. To improve the solubility, while retaining the inhibitory activity of WP1130 against Usp9x, we carried out structure-activity relationship (SAR) studies to map and define critical and useable chemical modifications to the parent compound. More than 220 compounds were designed, synthesized and assessed for Usp9x catalytic domain inhibitory activity (as previously described) and compared to WP1130 \(^2\). Those retaining catalytic domain inhibition were further screened for aqueous solubility, metabolic stability, pharmacokinetics, cellular activity and tumor vs. normal cell differential apoptosis. One compound emerged, [EOAI3402143 (G9), Fig.6A] that was 3-fold more effective against Usp9x catalytic activity than WP1130, had increased aqueous solubility and greater cellular Usp9x inhibitory activity than WP1130. G9 also demonstrated a therapeutic index of \(>10\), had sufficient metabolic stability and adequate pharmacokinetics to allow assessment of its \textit{in vivo} anti-tumor activity (Table 2, Supplemental Fig. S3). G9 displayed nM apoptotic activity against other myeloma cell lines and diffuse large B-cell lymphomas (Supplemental Fig. S4). G9 treatment of MM1.S cells resulted in the rapid onset and sustained inhibition of Usp9x and Usp24 (co-incident migration of both DUBs) activity (Fig. 6B). We confirmed Usp24 as a G9 target using HCT-116 Usp9x\(^{-/-}\) cells (Supplemental Fig. 3D). Other DUBs, such as Usp5, were also affected by G9 in these and other cell types as recently reported \(^2\). Dose-dependent inhibition of Usp9x/Usp24 and Usp5 were noted in G9 treated MM1.S cells (Fig. 6C), which was associated with a reduction in Mcl-1 levels, an increase in p53 stability and the rapid onset of apoptosis (cleaved PARP). We also noted a similar pattern of Usp9x inhibition and reduction of Mcl-1 levels in a freshly prepared G9-treated plasma cell leukemia (PCL) sample derived from a newly diagnosed patient (Fig. 6D). Interestingly, we did not detect Usp24 expression in this sample, but did note loss of Usp9x protein after G9 treatment, suggesting that Usp9x may be susceptible to decay in some cell types.

Mechanism of action studies suggests that G9 inhibits Usp9x (and Usp24) activity through a covalent, slowly reversible conjugation with Cys residues essential for substrate binding (unpublished observation). Further, \textit{in vitro} analysis demonstrated that 5 min incubation with compound is sufficient to mediate long-term Usp9x inhibition. Although G9 has a short half-life, sufficient peak levels were achievable to inhibit Usp9x activity \textit{in vivo}. Animals with subcutaneous MM1.S tumors were treated with G9 doses ranging from 2.5 – 10 mg/kg daily (i.p.) for 2 weeks. As shown in figure 7A, G9 doses at or above 5 mg/kg significantly suppressed tumor growth, noted as early as 3 days after initiating dosing. Animal weight was not adversely affected throughout the treatment interval (Fig. 7B). Tumor regrowth
was followed after discontinuation of G9 dosing (Supplemental Fig. S5A), which demonstrated a dose-dependent delay in resumption of tumor growth after stopping G9 treatment.

Additional studies were initiated to test higher doses and the impact of G9 on tumor Usp9x activity. As shown in figure 7C, tumor growth was significantly suppressed at all doses tested, with evidence for some tumor regression at the 15 mg/kg dose. A second trial of anti-tumor assessment at the 15 mg/kg G9 dose level confirmed evidence for tumor regression (Supplemental Fig. S5B). At the conclusion of the treatment interval, tumors were excised and photographed (Fig. 7D) and tumor lysates were assessed for DUB activity and blotted for several proteins. The results demonstrate that G9 suppressed Usp9x/Usp24 activity and reduces the protein expression levels of Usp9x, Usp24 and Mcl-1 in vivo (Fig. 7E). PARP cleavage was also activated by G9. Together, these results suggest that G9 targets Usp9x and Usp24 (as well as additional DUBs) in vivo and is able to safely reduce the growth or regress MM1.S myeloma tumors.

Discussion

In this study, we demonstrate that WP1130 effectively inhibits Usp9x in multiple B-cell malignancies with a wide spectrum of basal Usp9x expression or enzymatic activities. However, WP1130 is not absolutely Usp9x specific, which may not be a major concern as clinical evidence suggests that highly specific inhibitors are more likely to engage multiple resistance mechanisms. In addition, emerging data on the role of other cancer-associated DUBs supports the benefit of multi-target inhibition. As recently demonstrated, the WP1130 chemotype may be useful against various cancers with overexpressed or activated Usp9x.

Mcl-1 was previously reported to be a substrate of Usp9x in some tumors. We sought to confirm a direct role for Usp9x as a Mcl-1 regulator by assessing the response to Usp9x knockdown and identified several unexpected outcomes. Although short-term selection of Usp9X KD myeloma cells (shRNA and siRNA-mediated KD) demonstrated impact on cell survival, this effect was not sustained and did not appear to be fully dependent on Mcl-1. However, Usp9x KD caused sustained suppression of myeloma cell growth, as cells were unable to efficiently exit G2/M in the absence of Usp9x. These observations suggested that some Usp9x functions may be redundant and cellular adaptation may ensure cell survival in the absence of Usp9x. DUB activity measurements in chronic Usp9x KD cells or those with full genetic Usp9x disruption supported evidence of a role for Usp24 in that adaptation as these DUBs are closely related and Usp24 expression or activity were increased in Usp9x-deficient cells. Importantly Usp24 KD was more effective in controlling both myeloma cell survival and Mcl-1 protein levels, suggesting that
WP1130 activity against Mcl-1 is manifested through dual Usp9x/24 inhibition. Indeed, we have accruing evidence that both DUBs are capable of Mcl-1 association but the extent of the association may be controlled by more than recognition by the catalytic domain. Since the C-terminus of Usp9x was previously shown to bind other substrates, our mapping studies suggest that Mcl-1/Usp9x association may be subject to regulation by the extent of occupancy with other C-terminally bound Usp9x substrates. This may explain the previous inconsistencies associated with definition of Mcl-1 as a Usp9x substrate in cells of distinct origin. Interestingly, Mcl-1 binding maps to a domain distinct from that of other Usp9x substrates (i.e. Smurf1), suggesting that substrate recognition is not mediated by uniform Usp9x interactions. Further, the central Usp9x domain (aa 385-1593) appears critical for Mcl-1 binding, but finer mapping will be required to fully define critical contacts. This effort has been complicated by our inability to stably express central domain mutants of Usp9x. Interestingly, an N-terminal deletion mutant of Usp24 retained Mcl-1 and Usp9x binding activity, suggesting the existence of a Usp9x/Usp24/Mcl-1 complex. These observations, coupled with the observed effect of Usp24 KD on myeloma cell survival, support the possibility that Usp24 plays a central role in control of both Mcl-1 and Usp9x function and dual Usp9x/Usp24 inhibition may provide greater anti-tumor activity than more selective Usp9x or Usp24 inhibitors alone. Our preliminary studies have demonstrated that WP1130 blocks Usp9x activity by forming slowly reversible covalent adducts with cysteines configuring part of a substrate-binding zinc finger motif (Cys1771, Cys1774) within the catalytic domain of Usp9x. Usp24 retains a homologous zinc finger motif (Cys1859, Cys1863) which may underlie dual Usp9x/Usp24 inhibition by WP1130. This is a certain mechanistic departure from other DUB inhibitors that either directly target the active site Cys or interfere through binding at allosteric sites.

To exploit the Usp9x/Usp24 dual inhibitory activity of WP1130, we improved its activity and drug-like properties. EOAI3402143 (G9) was more effective than WP1130 in Usp9x inhibition and Mcl-1 destruction and had an improved therapeutic index against myeloma vs. normal CD34+ cells (Supplemental Fig. S3C). Although some improvement was achieved in selectivity, we were unable to fully modify DUB selectivity with G9 as we retained Usp5 inhibition which was previously noted as a WP1130 target. However, Usp5 was activated in myeloma cells and its inhibition increases p53 accumulation, as we and others have shown in the present study and other tumor types. Since p53 is infrequently inactivated by mutation in B-cell tumors, it remains a potential effector of apoptosis. Therefore, G9-mediated inhibition of Usp5 and p53 accumulation may amplify the effects of Usp9x/Usp24 inhibition in some tumors rather than pose a disadvantage due to partial selection.
Although G9 showed only an acceptable to moderate DMPK profile, this was overcome by taking advantage of the covalent character of G9, which included a dissociation of pharmacokinetics from pharmacodynamics and making quickly cleared compound more acceptable and even desirable due to a lower systemic exposure and reduced risk of idiosyncratic effects. Overall G9 had greater in vivo anti-tumor activity at lower doses than WP1130 and was well tolerated in animals (based on minimal loss of bodyweight). To investigate the pharmacodynamics of the compound, we have shown that Usp9x inhibition in vivo is sustained for up to 24 hours after G9 ip administration. We have seen dose-dependent tumor regrowth delays after stopping G9 treatment (Supplemental figure S5A), suggesting a slow recovery from reversible covalent inhibition. Further modifications of this current lead compound will result in an optimized candidate for detailed pre-clinical evaluation.

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Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


Figure legends.

Figure 1. WP1130 inhibits DUB activity and reduces Mcl-1 protein expression in myeloma cells.

A. Myeloma cells (as indicated) were treated with 5 μM WP1130 for 0, 2 and 4 h before assessing DUB activity by HA-UbVS labeling. The labeled DUBs were detected by immunoblotting with anti-HA antibody. HA labeled Usp9x is denoted. The protein lysates were also immunoblotted for total Ub, Usp9x, Mcl-1 and PARP. β-Actin was used as a protein loading control.

B. Z138 and Mino cells were treated as noted before cell lysates were screened for Usp9x activity (HA-labeled Usp9x), Usp9x, Mcl-1, PARP and Actin protein level by blotting.

C. Myeloma cells were treated with WP1130 alone, or pretreated (30 min) with proteasome inhibitors MG-132 (5 μM) or bortezomib (50 nM) prior to WP1130 treatment. Lysates were prepared after 4 hours and immunoblotted for Ub and Mcl-1. Equal load was confirmed by immunoblotting with β–Actin.

Figure 2. WP1130 inhibits DUB activity in primary B-cell tumors.

A. Top - Primary myeloma cells were treated with 5 μM WP1130 for 4 hours before assessing DUB activity as described in figure 1. The HA-labeled Usp9x protein is denoted. Middle - Total protein levels for Usp9x, Mcl-1 and β-Actin were also assessed by immunoblotting. Bottom - After 24 hours, aliquots of the same cells were subjected to staining with Annexin V-FITC/propidium iodide and analyzed by flow. Viability of untreated control cells was set at 100% and relative reduction in survival in WP1130 treated cells is noted below each blot.

B. Plasmacytoma cells from a newly diagnosed patient were treated with 0, 1.25, 2.5 and 5 μM WP1130 for 4 hours before measuring DUB activity by HA-UbVS labeling and the labeled DUBs were visualized by anti-HA immunoblotting. Mcl-1 and Actin protein levels were also measured by blotting. Viability of CD138+ cells was determined as described in A (above).

C. Primary CLL cells were left untreated or treated with WP1130 as described above. Patient CLL60 cells were treated with the WP1130 concentration indicated before assessing DUB activity, Usp9x, Mcl-1 and β-Actin expression by immunoblotting.

Figure 3. Usp9x knockdown reduces myeloma cell survival and proliferation and upregulates Usp24.

A. Immunoblot confirming Usp9x and Mcl-1 KD in H929 and MM1.S myeloma cells. Usp9x or Mcl-1 were knocked down by control or specific shRNA-retrovirus and selected for puromycin.
resistance before cell lysates were examined for the protein indicated (left) or cell survival by measurement of annexin positivity (right) The results represent the average +/- S.D. of triplicate assays. ** p-value <0.01, *** p-value <0.005.

B. Top - Equal number of scrambled control and Usp9x KD cells were plated on day 0 and cell numbers were assessed daily for 3 days. The numbers represent the average +/- S.D. of triplicate cell counts. Bottom – Cell cycle analysis was performed on control (red bars) and Usp9x KD (blue bars) cells obtained at day 3 following initial plating. Cell cycle phase was determined by propidium iodide staining and flow analysis and each value represents the average +/- S.D. of triplicate assessments. p-values are shown above each pair.

C. DUB activity was assessed (as described in figure 1) in control KD and Usp9x KD H929 cells left untreated or treated with 5 μM WP1130 for 4 hours. Usp9x, Mcl-1, PARP and Actin were also assessed by immunoblotting. HA-UbVS-labeled Usp9x and Usp24 are denoted (migration at similar molecular size).

D. Lysates from control and chronic Usp9x KD myeloma cells were subjected to immunoblotting for Usp9x, Usp24 and Actin.

E. Colon cancer cell lines with (Usp9x+/-) or without (Usp9x-/-) full Usp9x gene disruption were assessed for DUB activity by HA-UbVS labeling (top) of equal protein lysates. Lysates were also probed for Usp9x, Usp24 and β-Actin protein expression.

F. Equal protein lysates from myeloma cells were subjected to immunodepletion of the DUB indicated with anti-Usp9x or Usp24 (or control Ab) before measurement of total DUB activity by HA-UbVS labeling. The resultant loss of Usp9x or Usp24 from each supernatant was determined by immunoblotting.

G. Usp9x (left) and Usp24 (right) gene expression in myeloma patient samples was assessed by qPCR and compared to H929 control KD and Usp9x KD cells (green bars). Samples designated as high risk (red bars) or good risk (blue bars) of progressive disease based on their cytogenetic profiles are denoted. Each bar represents the average +/- S.D. of triplicate samples.

H. Usp9x and Usp24 protein expression was assessed by immunoblotting cell lysates derived from myeloma (H929) and mantle cell lymphoma (Z138) cell lines, normal tonsilar B-cells, or samples derived from two newly diagnosed or two relapsed myeloma patients. Actin was blotted as a protein loading control.

I. Tissue microarrays from normal tissue (spleen and tonsil) and confirmed pathologic samples from mantle cell lymphoma (MCL - left) or chronic lymphocytic leukemia (CLL - right) were
subjected to immuno-staining for Usp9x and Usp24. Examples of tumor samples with intermediate and high expression in the same specimen are shown. The Usp9x/Usp24 staining pattern in additional samples is shown in supplemental figure 2.

**Figure 4. Usp24 knockdown reduces Mcl-1 levels and myeloma cell survival.**

A. Usp24 or Mcl-1 were knocked down by control or specific shRNA-retrovirus and selected for puromycin resistance before cell lysates were examined for the protein indicated (left) or cell survival by measurement of annexin positivity (right). The results represent the average +/- S.D. of triplicate assays. *** p-value <0.005.

B. RPMI-8226 myeloma cell lines were subjected to siRNA-mediated knockdown of control (luciferase), Usp9x or Usp24 before assessing the protein indicated by immunoblotting (left). Increase in annexin positivity was assessed as a measure of cell viability. The values represent the average +/- S.D. of three replicates from two independent experiments. ** p-value <0.01, *** p-value <0.005.

**Figure 5. Usp9x and Usp24 associate with Mcl-1.**

A. HCT116 Usp9x+/+ (WT) and Usp9x−/− cell lysates were subjected to immunoprecipitation with normal IgG, anti-Mcl-1, anti-Usp9x or anti-Usp24 before immunoblotting the pulldown for Mcl-1, Usp24 or Usp9x (top 2 panels). H929 cell lysates were immunoprecipitated with normal IgG, anti-Mcl-1 or anti-Usp24 and immunoblotted for Mcl-1 or Usp24 (bottom panel).

B. Organization of the Usp9x and Usp24 constructs used in pulldown experiments and summary of their Mcl-1 binding activity. The position of the catalytic domain (UCH) is shown in shading. Flag sequence and HA epitope in the constructs are positioned at the N-termini. Numbers and letters designate highlighted amino acids.

C. Flag-tagged control or Flag-tagged full-length and deletion constructs of Usp9x (illustrated in B) were expressed in HEK293T cells and subjected to Flag immunoprecipitation followed by immunoblotting of Mcl-1.

D. Flag-tagged control or deletion constructs of Flag-E1-Usp9x retaining the active site cysteine (C1566) or a catalytic domain mutant (CDM – C1566A) were expressed in HEK293T cells and subjected to Flag pulldown, followed by Mcl-1 immunoblotting.

E. HEK293T cells were transfected with the constructs designated before the input lysates and anti-HA (Usp24) immunoprecipitates were subjected to immunoblotting with antibodies against Usp9x, Usp24 and Mcl-1.
Figure 6. Structure and activity of a novel DUB inhibitor.

A. Chemical structures of WP1130 and EOAI3402143 (also designated as G9).

B. MM1.S cells were treated as described with 5 μM G9 before assessing DUB activity (top) and total protein expression of Usp9x, Usp24, Mcl-1, PARP and β-Actin. The HA-labeled Usp9x/Usp24 band is shown at the top.

C. MM1.S cells were treated with the G9 concentration indicated before assessing changes in DUB activity associated with Usp9x/Usp24 and Usp5. Lysates were also immunoblotted for Mcl-1, p53 and cleaved PARP as a measure of apoptosis. Actin was blotted as a loading control.

D. Cells derived from a plasma cell leukemia patient (PCL) were treated with G9 for the time indicated before DUB activity and specific protein levels were determined by HA-UbVS labeling and immunoblotting.

Figure 7. In vivo anti-myeloma activity of a DUB inhibitor.

A. NSG mice with equally size-matched dorsal MM1.S tumors were treated with the indicated dose of G9 every day for 2 weeks. Tumor size was measured at the time points indicated. Each bar represents the average +/- S.D. of tumor size from 5 animals. p-values are designated within the figure.

B. Animal body weight was measured in control and G9 treated mice from figure 7A. Body weight was measured at the interval noted and each bar represents the average +/- S.D. from 5 mice per group.

C. MM1.S tumors growing in NSG mice (3/group) were treated daily with the indicated concentration of G9. Tumor volumes at each designated interval were recorded. The average tumor volume +/- S.D. from three mice per group is shown. p-values are the same as described in figure 7A.

D. Tumors from figure 7C were excised at the conclusion of the study (day12) and photographed.

E. One hour after the final G9 treatment, tumors from control and G9 treated (15 mg/kg) mice were excised, quick frozen in liquid nitrogen and subjected to lysis. Equal protein lysates from these tumors was subjected to DUB activity assessment (top; as in figure 1A) and immunoblotted for the protein indicated. The top band represents HA-UbVS-labeled Usp9x and Usp24 (co-migrating proteins).
Table 1. Clinical and cytogenetic characteristics of patients whose tumor samples were evaluated in this study.

<table>
<thead>
<tr>
<th>MM Patient ID</th>
<th>Time Since Diagnosis</th>
<th>Treatment Regimens</th>
<th>Response to Last Tx</th>
<th>Poor Cytogenetic Prognoses</th>
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</thead>
<tbody>
<tr>
<td>6821</td>
<td>6 years</td>
<td>VelDoxDex, Bexxar, Thal Maint, ASCT</td>
<td>Very Good Partial Response followed by Relapse Absent</td>
<td></td>
</tr>
<tr>
<td>7261</td>
<td>4 years</td>
<td>VelDexThal-PACE, Bexxar, Tandem ASCT, VelDexThal, RevVelDex, CytRevVelDex</td>
<td>Progressive Disease</td>
<td>ND</td>
</tr>
<tr>
<td>5500</td>
<td>New Diagnosis of Plasmacytoma and Plasma Cell Leukemia</td>
<td>48 hrs of Radiation</td>
<td>Naïve Disease 17p and 13 del, t(14;16)</td>
<td></td>
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<tr>
<td>5281</td>
<td>New Diagnosis of MM</td>
<td>None</td>
<td>Naïve Disease del 13 and t(4;14)</td>
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<tr>
<td>5609</td>
<td>7 years</td>
<td>VelDoxDex, ASCT</td>
<td>Progressive Disease</td>
<td>Trisomy 3 and 9, 13 del</td>
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<tr>
<td>4283</td>
<td>5 years</td>
<td>RevVelDoxDex, CyBorD</td>
<td>Stable Disease</td>
<td>Trisomy 3, 7, 9, 11, and 15, 14q32 del</td>
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<tr>
<td>0823</td>
<td>New Diagnosis of Plasma Cell Leukemia</td>
<td>None</td>
<td>Naïve Disease Normal</td>
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Table 2. Comparison of compound solubility, activity, microsomal metabolism, initial pharmacokinetics and toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WP1130</th>
<th>EOAI3402143</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous Solubility [μM]</td>
<td>2.3</td>
<td>80 (HCl salt)</td>
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<tr>
<td>Enzyme IC50 (Usp9x Cat. Dom.) [μM]</td>
<td>4.8</td>
<td>1.6</td>
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<tr>
<td>Liver Microsomes t1/2 [min]</td>
<td>&gt;60</td>
<td>40</td>
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<tr>
<td>Mouse PK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV (20 mg/kg) t1/2 [min]</td>
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<td>45</td>
</tr>
<tr>
<td>ng/ml (0.5 hours)</td>
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<td>1980</td>
</tr>
<tr>
<td>ng/ml (4 hours)</td>
<td>56</td>
<td>193</td>
</tr>
<tr>
<td>IP (20 mg/kg) t1/2 [min]</td>
<td>90</td>
<td>30-45</td>
</tr>
<tr>
<td>ng/ml (0.5 hours)</td>
<td>215</td>
<td>1920</td>
</tr>
<tr>
<td>ng/ml (4 hours)</td>
<td>119</td>
<td>120</td>
</tr>
<tr>
<td>Toxicity (LD50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV [mg/kg]</td>
<td>&lt;30</td>
<td>30</td>
</tr>
<tr>
<td>IP [mg/kg]</td>
<td>40</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>
Figure 1

A. DUB Activity (HA Blot) and Blot images for MM1.R, H929, MM1.S, and RPMI 8226.

B. Western blot analysis of HA-labeled Usp9x, Usp9x, Mcl-1, PARP, Cleaved PARP, Z138, and Mino after 5 μM WP1130.

C. Western blot analysis of Ub, Mcl-1, and Actin for MM1.R and RPMI 8226.
Figure 6

A. WP1130

EOAI3402143 (G9)

B. MM1.S

C. MM1.S

D. MM1.S
Figure 7

A. Tumor Volume (mm$^3$) vs. Days of G9 Treatment

B. Animal Weight (g) vs. Days of G9 Treatment

C. Tumor Volume (mm$^3$) vs. Days of Treatment

D. Images of tumors after G9 treatment

E. Western Blot Analysis for DUB Activity

* $p<0.05$
** $p<0.005$
*** $p<0.0002$
Targeting deubiquitinase activity with a novel small molecule inhibitor as therapy for B-cell malignancies

Luke F. Peterson, Hanshi Sun, Yihong Liu, Harish Potu, Malathi Kandarpa, Monika Ermann, Stephen M. Courtney, Matthew Young, Hollis D. Showalter, Duxin Sun, Andrzej Jakubowiak, Sami N. Malek, Moshe Talpaz and Nicholas J. Donato