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Blockade of Deubiquitylating Enzyme USP1 Inhibits DNA Repair and Triggers Apoptosis in Multiple Myeloma Cells

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Abstract

Purpose—The ubiquitin proteasome pathway is a validated therapeutic target in multiple myeloma (MM). Deubiquitylating enzyme USP1 participates in DNA damage response and cellular differentiation pathways. To date, the role of USP1 in MM biology is not defined. In the present study, we investigated the functional significance of USP1 in MM using genetic and biochemical approaches.

Experimental Design—To investigate the role of USP1 in myeloma, we utilized USP1 inhibitor SJB3-019A (SJB) for studies in myeloma cell lines and patient MM cells.

Results—USP1-siRNA knockdown decreases MM cell viability. USP1 inhibitor SJB selectively blocks USP1 enzymatic activity without blocking other DUBs. SJB also decreases the viability of MM cell lines and patient tumor cells, inhibits bone marrow plasmacytoid dendritic cells-induced MM cell growth and overcomes bortezomib-resistance. SJB triggers apoptosis in MM cells via activation of caspase-3, caspase-8 and caspase-9. Moreover, SJB degrades USP1 and downstream inhibitor of DNA binding proteins, as well as inhibits DNA repair via blockade of Fanconi anemia pathway and homologous recombination. SJB also downregulates MM stem cell renewal/survival-associated proteins Notch-1, Notch-2, SOX-4 and SOX-2. Moreover, SJB induced generation of more mature and differentiated plasma cells. Combination of SJB and HDACi ACY-1215, bortezomib, lenalidomide, or pomalidomide triggers synergistic cytotoxicity.

Conclusions—Our preclinical studies provide the framework for clinical evaluation of USP1 inhibitors, alone or in combination, as a potential novel MM therapy.

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Conflict-of-interest disclosure KCA is on Advisory board of Celgene, Millenium, Gilead, and Bristol Myers Squibb, and is a Scientific Founder of Acetylon, Oncopep, and C4 Therapeutics. The remaining authors declare no competing financial interest.

Keywords

Multiple Myeloma; USP1; Deubiquitylating enzymes; DNA repair; Pre-clinical study

Introduction

Proteasome inhibitors are effective therapy for relapsed/refractory, relapsed, and newly diagnosed multiple myeloma (MM); however, the development of resistance and relapse of disease is common¹⁻³. Recent research discovered novel drugs that modulate protein ubiquitin-conjugating/deconjugating enzymes rather than the proteasome itself. We recently showed that targeting deubiquitylating enzymes (DUBs) USP14, UCHL5, and USP7 can overcome proteasome inhibitor resistance in MM⁴⁻⁸.

DUBs deconjugate ubiquitin from targeted proteins and facilitate regeneration of free ubiquitin pools⁴. Additionally, DUBs maintain cellular protein homeostasis by modulating protein activation, turnover rate, recycling, and localization⁴. Alteration in DUBs activity has been linked with several diseases, including cancer^{5, 6}. The human genome encodes about 100 DUBs, which are classified into five families: the USP (the ubiquitin-specific processing protease), UCH (the ubiquitin C-terminal hydrolyase), OTU (the ovarian tumour), MJD (the Josephin domain) and JAMM (the Jab1/Mov34 metalloenzyme). The first four families are cysteine proteases, whereas the fifth family are metalloproteases, and to date USP and UCH are the best characterized families⁴.

USP1 regulates DNA repair through Fanconi anemia pathway by deubiquitylating DNA repair proteins, FANCD2-Ub and PCNA-Ub⁹. For example, USP1 deubiquitylates mono-ubiquitylated PCNA, which inhibits recruitment of DNA polymerases in the absence of DNA damage, and thereby leads to regulated DNA repair. USP1 also regulates DNA break repair via homologous recombination pathway¹⁰. Conversely, inhibition of USP1 sensitizes cancer cells to chemotherapy and radiation⁹. Since USP1 participates in DNA damage response pathways, USP1-knockout mice are genetically unstable and highly sensitive to DNA damage¹¹. Finally, USP1 inhibits cell differentiation by stabilizing tumor-promoting inhibitor of DNA binding (ID) proteins^{12, 13}. To date, the role of USP1 in MM biology is undefined. In the present study, we investigated the functional significance of USP1 in MM using genetic and biochemical approaches.

Materials and methods

Cell culture and reagents

MM cell lines and normal donor-derived PBMCs were cultured in complete medium containing 10% FBS and antibiotics. All cell lines were tested for mycoplasma contamination using MycoAlert™ mycoplasma detection kit (Lonza, Basel, Switzerland). Plasmacytoid dendritic cells (pDCs), BMSCs, or tumor cells from MM patients were purified and cultured as described previously¹⁴. All patient samples were obtained with prior informed consent in accordance Helsinki protocol. Bone marrow MNCs were purchased

from Allcells (USA). SJB3-019A, Bortezomib, Lenalidomide, Pomalidomide and ACY-1215 were obtained from Selleck chemicals (USA).

Cell cycle, Cell viability, and apoptosis assays

Cell cycle analysis was performed as described previously¹⁵. Cell viability was assessed by WST-1/CellTiter-Glo (CTG) Luminescent assays, as in prior study¹⁶. Apoptosis was measured by Annexin/PI staining.

Western blotting assays

Immunoblotting was performed as previously described¹⁵ using antibodies against USP1, USP2, USP5, USP7, USP14, Caspase-3/8/9, p21, FANCD2, FANCI, PCNA, Rad51, GAPDH (Cell Signaling, Beverly, MA, USA); ID1, ID2, ID3, ID4, Notch-1, Notch-2, Sox-4 and Sox-2 (Bethyl Laboratories, Montgomery, TX, USA).

Transfection assays

MM.1S cells were transiently transfected with control scr-siRNA or USP1-siRNA using the cell line Nucleofector kit V (Amaxa Biosystems, Cologne, Germany). Cells were harvested 24h post-transfection, followed by analysis using both immunoblotting and cell viability assays.

Ubiquitin vinyl sulfone (Ub-VS) labeling, Ub-AMC, and Tetra-ubiquitin chain cleavage assays

Cells were treated with or without SJB for 3h; cells were harvested and lysed. Total protein (25µg) was labeled with HA-linked Ub-VS probe (1µM) for 30 mins at 37°C, and analyzed with immunoblotting.

Ub-AMC assay

Recombinant DUBs (USP1/UAF1, USP2, USP5, USP7 or UCH37) were incubated with SJB for 30 mins at 37°C, and then UB-AMC was added for another 30 mins, followed by measurement of fluorescence intensity. **Ubiquitin-linked K48 Chain cleavage assay** Purified rDUBs were incubated with SJB for 30 mins, followed by the addition of K-48 linked tetra ubiquitin chains. The reaction was terminated after 30 mins by addition of reducing buffer, and samples were analyzed by western blotting¹⁷.

Immunostaining

MM cells were stained with Rad51 Ab and giemsa stain as described previously, and sections were then imaged by microscopy¹⁸.

USP1 gene expression analysis

The exon-1.0 ST array data for 170 newly diagnosed MM patients were quality controlled and normalized with aroma affymetrix package. Gene expression was estimated with a PLM model. The survival analysis was carried out using the R-package “Survival”. The raw data for expression profiling and the CEL files can be found at the website Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers: GSE5900 and

GSE2658. Survival data can be accessed at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39754>

Statistical analysis

Student's *t* test was utilized to derive statistical significance. Synergistic cytotoxic activity of combination regimes was assessed with isobologram analysis and CalcuSyn software program¹⁹.

Results

USP1 expression analysis in MM cells

Examination of Gene expression datasets showed a higher *USP1* in clonal plasma cells from patients with MGUS (Monoclonal Gammopathy of Undetermined Significance) SMM (Smoldering MM), and active MM versus normal plasma cells (Figure 1A). Immunoblot analysis showed elevated USP1 levels in a panel of MM cell lines versus normal healthy donor-derived bone marrow MNCs or PBMCs (Figure 1B). The prognostic relevance of *USP1* was assessed by correlating baseline *USP1* expression in BM biopsy samples with overall and event-free survival of 170 MM patients. All patients analyzed in this study were newly diagnosed and no therapy was administered at the time of expression profiling²⁰. We found a statistically significant inverse correlation between USP1 levels and both overall and event-free survival (Figure 1C). These findings indicate a role of USP1 in MM pathogenesis.

To determine the functional significance of USP1 in MM, we performed loss-of-function studies with USP1-small interfering RNA. Transfection of USP1-siRNA, but not scr-siRNA, reduces MM cell viability (Figure 1D, bar graph; $p < 0.05$). These data show a role for USP1 in survival of MM cells.

Biochemical Characterization of USP1 inhibitor SJB3-019A (SJB)

SJB, 2-(pyridin-3-yl)naphtho[2,3-d]oxazole-4,9-dione, targets USP1 in an irreversible manner (Figure 2A). We utilized Ub-AMC (ubiquitin 7-amino-4-methycoumar) assays (Figure 2B) to assess the effect of SJB on USP1 or other DUBs using both recombinant DUB (rDUBs) proteins and cellular DUBs. *In vitro* assays using purified rDUBs showed that SJB inhibits USP1 (>90% inhibition at 1 μ M) (Figure 2C). Importantly, SJB did not significantly affect the activity of other rDUBs (USP2/USP2/USP5/USP7/USP14 or UCH37) (Figure 2C and 2D). Moreover, a USP7 inhibitor P5091 did not inhibit USP1 DUB activity (Figure 2C). ML323 was used as a positive control for USP1 inhibition assays (Supplementary Figure 1).

We next examined the effect of SJB against cellular DUB enzymatic activity. SJB blocked cellular USP1 activity in a concentration-dependent manner, without markedly affecting activity of other DUBs (USP2, USP7, USP14) (Figure 2E, bar graph). A higher concentration of SJB (1 μ M) modestly inhibited USP5 activity. Immunoblot shows equivalent USP1-immunoprecipitates were analyzed for DUB activity assay (Figure 2E). Together, these data suggest that SJB is a specific inhibitor of USP1.

To ascertain the effect of SJB on DUB activity, we performed competitive labeling between SJB and ubiquitin-active site probe HA-Ub-VS. Untreated cell lysates incubated with Ub-VS probe showed Ub-VS-USP conjugate formation, represented by an increase in mass of USPs of ~10kDa (Figure 3A). Importantly, this conjugate formation was inhibited in the presence of SJB for USP1 enzyme, but not other DUBs. Immunoblotting with anti-HA Ab showed equal protein loading of control and treated protein lysates (Supplementary Figure 2). These data suggest that SJB binds to cellular USP1 and blocks its deubiquitylating activity. Furthermore, analysis using K48-linked Ub-tetramer chain cleavage assays showed that SJB inhibits USP1 mediated Ub chain disassembly in a concentration-dependent manner (Figure 3B). Proteasome inhibitor marizomib served as negative control in these assays. No significant inhibitory effect of SJB was noted in USP7 or USP2 mediated Ub chain disassembly (Supplementary Figure 3). Taken together, these findings provide evidence for the specificity of SJB against USP1.

Cytotoxic activity of SJB against MM

SJB reduces the viability of all MM cell lines in a concentration-dependent manner (IC_{50} ranges from 100–500 nM) (Figure 4A). Treatment of purified MM cells from patients showed a concentration-dependent decrease in the viability of all patient MM cells (Fig 4B). In contrast, no significant effect of SJB was noted against normal PBMCs (Fig 4C). However, the highest concentration of SJB (1 μ M) decreases viability of PBMCs by 10%–20%. These data suggest a favorable therapeutic index for SJB in MM. A comparative analysis of SJB with other reported USP1 inhibitors (ML323, SJB2-043, pimozone, GW7647) showed a more potent and selective anti-MM activity of SJB (Supplementary Figure 4).

SJB activity in the MM BM microenvironment

Previous studies have shown that BMSCs mediate MM cells growth and block drug-induced cytotoxicity^{21–24}. Treatment of patient BMSCs for 24h with SJB does not decrease their viability. Importantly, SJB significantly inhibits BMSCs-induced MM.1S cell growth (Fig 4D). Our earlier reports highlighted the growth-promoting role of pDCs in the MM BM microenvironment^{14, 25, 26}. Using pDC-MM co-culture model, we found that SJB inhibits pDCs-induced MM.1S cell growth (Fig 4E). These data demonstrate that SJB targets MM cells even in the cytoprotective MM-host BM microenvironment.

Mechanistic insight into the SJB activity

Cell cycle analysis showed that SJB induces G1 phase growth arrest associated with decrease in G2/M and S phase (Fig 5A). In concert with these findings, SJB upregulated G1-associated cell cycle protein p21 (Fig 5A). Moreover, treatment of MM.1S and Dox-40 cells with SJB induced significant apoptosis associated with a marked increase in PARP cleavage, caspase-3, caspase-8, and caspase-9 (Fig 5B and Supplementary Figure 5A).

SJB disrupts homologous recombination (HR) in MM cells

USP1 regulates DNA repair and Fanconi anemia (FA) pathways through its association with binding partner UAF1²⁷. In particular, USP1 regulates DNA damage response by

deubiquitylation of DNA repair proteins, Ub-FANCD2 and Ub-PCNA⁹. We therefore examined whether SJB-mediated inhibition of USP1 affects FA signaling pathway in MM cells. MM.1S cells were treated with SJB for 12h, and cellular levels of Ub-FANCD2, Ub-FANCI and Ub-PCNA were then analyzed by western blotting. As shown in Figure 5C, USP1 inhibition by SJB led to an increase in Ub-FANCD2, Ub-FANCI, and Ub-PCNA levels (Fig 5C).

Previous studies showed that USP1 inhibition affects homologous recombination (HR)¹⁰, and we next examined the effects of SJB on HR in MM cells. For these studies, we assessed whether SJB alters expression of HR-associated RAD51 protein²⁸. RAD51 accumulates at discrete foci on chromosomal DNA during meiotic prophase; and importantly, DNA damage triggers RAD51 foci formation²⁸. Our study showed high levels of RAD51 protein in MM cells, indicating ongoing DNA damage in these cells²⁹. Treatment of MM cells with SJB decreased RAD51 foci formation, assessed by immunofluorescence (IFC) studies (Figure 5D). Quantification of IFC data showed decreased RAD51 foci/nuclei in SJB-treated MM.1S cells (Figure 5D). Together, these findings suggest that SJB abrogates HR mechanisms in MM cells.

We next determined whether the cytotoxic effects induced by SJB are irreversible in drug-washout experiments. MM.1S cells were treated with SJB for a short interval (4h); cells were then washed to remove SJB and cultured in complete medium for another 24h, followed by analysis of cell viability. Results showed that short-term (4h) treatment with SJB triggers significant cytotoxicity in MM.1S cells (Figure 5E). Although the extent of MM.1S cell death triggered by short-term (4h) exposure to SJB is less than long-term continuous treatment, it is sufficient to block DNA repair- and HR-related signaling pathways, as well as induce cytotoxicity.

SJB induces the morphological differentiation and maturation of MM cells by blocking USP1-associated downstream signaling via ID proteins

Inhibitor of DNA binding (ID) proteins belong to a class of helix-loop-helix (HLH) family of transcriptional regulatory proteins, which consists of four members (ID1-ID4)³⁰. ID proteins inhibit bHLH protein-mediated transactivation of genes involved in cellular differentiation pathways³⁰. Importantly, ID1 is downstream target of USP1³¹. Treatment of MM.1S cells with SJB decreased USP1 and the ID1 protein levels (Figure 6A). Besides ID1, SJB treatment also decreased other ID protein family members ID2, ID3, and ID4 (Figure 6A). Treatment of resistant cell line Dox-40 slightly reduced USP1 levels and did not affect ID protein levels (Supplementary Figure 5B). Importantly, GEP analysis of ID1 and ID2 proteins showed an inverse correlation with survival in MM patients (Supplementary Figure 6).

Earlier studies showed that ID proteins are highly expressed in various cancer types including MM, and promote stem cell survival^{30, 32}. Since SJB downregulates ID proteins, we examined whether SJB affects differentiation of MM cells using Giemsa staining assays. A significantly decreased nuclear-cytoplasmic ratio was observed in SJB-versus DMSO/control-treated MM.1S cells (Figure 6B). SJB-treated cells showed a more mature and differentiated state versus untreated cells: immature plasmablasts with large nuclei in

untreated cells (Figure 6B left panel) versus condensed nuclei with rough chromatin pattern and a more prominent cytoplasm in SJB-treated cells (Figure 6B right panel). These data indicates that SJB induces plasma cell differentiation with prominent cytoplasm and a reduced nuclear-cytoplasmic ratio.

Previous studies showed ID proteins contribute to chemoresistance and cancer stemness³². For example, ID4 regulates the expression of stemness-associated Sox2- and Notch-signaling in gliomas cells^{33, 34}. We found that SJB-mediated inhibition of USP1/ID signaling led to downregulation of stemness-associated Sox-4/Sox-2/Notch-1/Notch-2 signaling proteins in MM cells (Figure 6C). Additionally, Notch signaling promotes the maintenance and proliferation of hematopoietic stem cells³⁵. Previous studies have reported presence of MM clonogenic side population (MM-SP) with characteristic stem like features³⁶. We therefore here evaluated the effect of SJB on MM-SP using flow cytometry analysis of Hoechst 33342 stained cells. SJB significantly (38% decrease) lowered the fraction of MM-SP (Figure 6D). Verapamil and Reserpine served as positive controls. Importantly, SJB exhibited cytotoxic activity against MM-SP.

Combining SJB with bortezomib, lenalidomide, pomalidomide or the HDAC6 inhibitor ACY-1215 induces synergistic cytotoxicity

Preclinical studies laid the framework for clinical trials of bortezomib in combination with other anti-MM agents³⁷. Isobologram analysis for synergistic anti-MM activity showed that the combination of SJB and bortezomib triggers synergistic anti-MM activity (Supplementary Figure 7A). The mechanism of synergy between SJB and bortezomib may include enhanced accumulation of ubiquitylated proteins, which are not degraded and triggering of distinct cell-death signaling pathways.

Protein degradation also utilizes HDAC dependent aggressome-autophagic cell death-signaling pathway. Interestingly, the combination of SJB and HDAC6 inhibitor ACY-1215 triggers synergistic anti-MM cytotoxicity (Supplementary Figure 7B). Similarly, we found that combination of SJB and lenalidomide (Supplementary Figure 7C) or pomalidomide (Supplementary Figure 7D) triggered synergistic cytotoxicity against MM cells. These combination studies were also performed using primary MM tumor cells (Supplementary Figure 8). These data provide the framework for scientifically-informed clinical trials combining strategies to inhibit USP1 with standard-of-care anti-MM agents.

Discussion

We utilized MM cell lines, patient cells, and co-culture models of MM cells with BMSCs or pDCs, as well as genetic and biochemical and strategies, to validate deubiquitylating (DUB) enzyme USP1 as a therapeutic target in MM. USP1 is highly expressed in MM cells compared to normal cells. A similar high USP1 expression was shown in melanoma, sarcoma, cervical, and gastric cancers^{9–12}. *USP1* expression correlates with poor prognosis in MM patients.

RNA interference and biochemical strategies were utilized to determine the functional significance of USP1 in MM. USP1-siRNA decreases MM cell viability. We utilized a

pharmacological inhibitor of USP1 SJB3-019A (SJB) that targets USP1³⁰. The specificity of SJB was confirmed by various experiments: first, we show that SJB potently and selectively block USP1 activity without inhibiting other DUBs (USP2/USP5/USP7/USP14/UCH37); second, SJB inhibited binding of USP1 with HA-Ub-VS probe, but it did not affect labeling of other DUBs with probe; and third, SJB inhibited USP1, but not USP2 or USP7, triggered cleavage of ubiquitin tetramer chains.

A recent study demonstrated that USP1 inhibition induces apoptosis in leukemic cells³⁰. Here we show that SJB triggers apoptosis in MM cells *in vitro*, without reducing the viability of normal PBMCs. Moreover, SJB triggered more potent anti-MM activity versus other available USP1 inhibitors (ML323, SJB2-043, Pimozide, GW7647)³¹. Of note, we found that SJB retains its activity against MM cell lines resistant to conventional and novel therapies. Distinct genetic backgrounds³⁸ and/or drug resistance characteristics may account for the differences in IC₅₀ of SJB against MM cell lines. Differential USP1 expression was observed among various MM cell lines; however, we found no direct correlation between USP1 expression and sensitivity to SJB. Other factors, such as expression/function of USP1 binding partners (UAF1) or USP1 downstream signaling proteins, may impact the overall potency of SJB against MM cell lines. Our data in MM model are consistent with cytotoxic effects of USP1 inhibition observed in leukemic cells³¹.

We next assessed whether SJB can overcome bortezomib-resistance in MM cells. For these studies, we utilized bortezomib-sensitive (ANBL6.WT) and -resistant (ANBL6.BR) MM cell lines³⁹. SJB induced cytotoxicity in ANBL6.BR cells, which confirmed the ability of SJB to overcome bortezomib-resistance. Additionally, SJB was active against tumor cells from patients with MM resistant to novel (bortezomib, lenalidomide), conventional (dexamethasone) agents. The BM microenvironment (BMSCs and pDCs) promotes MM cell growth, survival, and drug resistance; importantly, SJB triggers apoptosis in MM cells even in the presence of the BM milieu.

USP1-UAF1 regulates DNA damage response signaling pathways⁹. We examined the effect of SJB on the three essential DNA repair pathways associated with USP1⁹: 1) Fanconi anemia (FA) pathway (via FANCD2/FANCI); 2) Translesion synthesis (via PCNA); and 3) DNA double strand break repair through homologous recombination (via RAD51). USP1-UAF1 deubiquitylates FANCD2 and FANCI proteins; conversely, knockdown of USP1 results in elevated Ub-FANCD2 and Ub-FANCI levels, which in turn disrupts FA repair pathway³¹. We found that USP1 inhibition by SJB increased the levels of both Ub-FANCD2 and Ub-FANCI in MM cells. These findings suggest that SJB inhibits the FA repair pathway in MM cells, and that SJB may sensitize MM cells to DNA damaging anti-MM therapies. Indeed, a recent study using a non-small cell lung cancer model showed that USP1 inhibitor ML323 sensitizes cisplatin-resistant cells to cisplatin^{31, 40}. Additionally, USP1 plays a role in homologous recombination (HR)-mediated DNA repair, and USP1 blockade disrupts this pathway. Here we found that MM cells express high levels of HR-associated DNA repair protein RAD51. Treatment of MM cells with SJB significantly decreased RAD51 foci formation, suggesting that SJB blocks activation of HR-mediated DNA repair pathways. These findings are consistent with our washout experiments showing that even short treatment duration (4h) of MM cells triggers significant cytotoxicity (Figure 5E). Together,

the ability of SJB to block multiple DNA repair mechanisms likely contributes to its overall potent anti-MM activity.

Besides DNA repair proteins, USP1 also promotes stabilization of ID (ID1-ID4) proteins³¹. ID proteins are highly expressed in proliferating cells and promote stem cell-like characteristics in osteosarcoma cells^{9,12}. Studies in MM have shown that histone methyltransferase protein MMSET promotes oncogenic transformation by transcriptional activation of ID1 protein expression⁴¹. In our study, we found that SJB downregulates ID1, ID2, ID3, and ID4 proteins in MM cells, suggesting that it may affect the differentiation state of MM cells and/or MM stem cell-like cell populations. A prior study showed that subpopulations of MM with distinct morphology and immunophenotype are consistent with distinct phases of differentiation⁴². Additionally, the presence of immature MM cells portends poor prognosis, and several studies have reported the survival advantage for patients with plasmacytic versus plasmablastic type MM⁴²⁻⁴⁴. In our study, we found that SJB induced MM cell differentiation and maturation at low concentrations, evidenced by well-developed cytoplasm with reduced nuclear-cytoplasmic ratio. These findings suggest that USP1 inhibition may represent a potential differentiation therapy in MM.

The modulation of ID proteins by USP1 inhibitor SJB has further biologic implications and clinical applications. Prior studies have shown that ID proteins function as master regulators of stem cell identity³⁰. Specifically, loss of ID proteins affects both the self-renewal and the tumor-initiating capacity of cancer stem cells in colorectal cancer and gliomas^{33, 45}. Conversely, ID4 relieves miR-9 mediated suppression of SOX-2 and enhances stem cell in gliomas⁴⁶; and overexpression of ID4 induces activation of Notch signaling and stem cells in gliomas³⁴. In our study, we showed that SJB inhibits SOX and Notch signaling in MM cells. Notch1 and Notch2 are highly expressed in primary MM cells and their expression increases with progression from MGUS to MM³⁵. Moreover, overexpression of notch receptors/ligands are integral to MM stem cell self-renewal and proliferation³⁵. Finally, *SOX2* expression is a key feature of clonogenic MM cells^{47, 48}. Importantly, we here found that inhibition of USP1/ID/Notch/Sox2 pathway by SJB decreased the percentage of clonogenic MM side population (MM-SP). Taken together, our data show that: 1) SJB triggers immature plasma cells to differentiate, mature, and undergo apoptosis; and 2) SJB decreases MM stem cell-like clonogenic population (MM-SP).

Finally, we also examined whether SJB enhances the anti-MM activity of other agents. Isobologram analysis for synergistic anti-MM activity demonstrated that the combination of SJB with bortezomib, HDAC6i ACY1215, lenalidomide or pomalidomide triggers synergistic anti-MM activity. The mechanism of synergy between SJB and other anti-MM agents is associated with activation of distinct apoptotic signaling pathways as well as blockade of DNA repair mechanisms. Future studies for SJB will be done using *in vivo* assays. Our preclinical data support clinical investigation of USP1 inhibitors in MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key points

1. Deubiquitylating enzyme USP1 is highly expressed in MM and correlates with poor patient survival.
2. USP1 inhibition using genetic and pharmacological strategies triggers apoptosis and overcomes bortezomib-resistance.

Translational Relevance

Proteasome inhibitors are effective therapy for multiple myeloma (MM); however, the development of resistance and relapse of disease is common. Recent research discovered novel drugs that modulate protein ubiquitin-conjugating/deconjugating enzymes rather than the proteasome itself, thus leading to less toxic side effects and can overcome proteasome inhibitor resistance in MM. In this study, we show that USP1 inhibition by SJB3-019A decreases cell viability in MM cells. The modulation of ID proteins and DNA repair proteins by USP1 inhibitor SJB3-019A shown here has further biologic implications and clinical applications. SJB3-019A mediated inhibition of USP1/ID/Notch/Sox2 pathway triggers immature plasma cells to differentiate, mature, and undergo apoptosis. Isobologram analysis demonstrated that the combination of SJB3-019A with bortezomib, HDAC6i ACY1215, lenalidomide or pomalidomide triggers synergistic anti-MM activity. Our preclinical data support clinical investigation of USP1 inhibitors alone or as in combination with other agents in MM.

Figure 1. USP1 in MM

(A) *USP1* expression in plasma cells from normal healthy donors, as well as tumor cells from patients with MGUS, SMM, and MM. Expression data: GSE5900 and GSE2658 from GEO (<https://www.ncbi.nlm.nih.gov/geo/>) Normal-MM and normal-SMM *USP1* expression p values are 0.0005 and 0.002 respectively (B) Purified peripheral blood mononuclear cells (PBMCs), bone marrow MNCs from normal donors and MM cell lines were assessed for *USP1* by immunoblotting with anti-*USP1* and anti-GAPDH antibodies. (C) Kaplan-Meier plots of *USP1* expression versus overall and event free survival of MM patients. Red line indicates patient group with higher *USP1* expression, and blue line show patient cohort with lower *USP1* expression. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39754> (D) MM.1S cells were transfected with genome control-siRNA/scr-siRNA or *USP1*-siRNA and cultured for 24h, followed by analysis of cell viability by WST assay. Percent cell viability was normalized against scr-siRNA control (mean $\pm$ SE; $p < 0.005$, $n=3$). Western blot shows *USP1* expression in cells transfected with Scr-siRNA or *USP1* siRNA.

Figure 2. Biochemical Characterization of USP1 inhibitor SJB3-019A (SJB)

(A) Chemical structure of SJB3-019A: 2-(pyridin-3-yl) naphtho[2,3-d]oxazole-4,9-dione. (B) Schematic representation of Ub-AMC assay. DUB removes ubiquitin from its substrate Ub-AMC, and fluorescent AMC is measured. (C) Recombinant USP1/UAF1 complex or rUSP2 were incubated with DMSO control, USP1 inhibitor SJB, or USP7 inhibitor P5091 for 30 mins at 37°C, followed by the assessment of DUB activity using Ub-AMC assay (mean $\pm$ SE; $p < 0.05$ for USP1 activity in control versus SJB treated samples, $n=3$). (D) Indicated rDUBs were incubated with DMSO control or USP1 inhibitor SJB for 30 mins at 37°C, followed by assessment of DUB activity using Ub-AMC assay (mean $\pm$ SE; $p < 0.05$, $n=3$). (E) MM.1S cells were treated with DMSO control or SJB for 3h; protein lysates were subjected to immunoprecipitation with different DUBs (USP1, USP2, USP5, USP7 and USP14), followed by analysis of DUB expression and enzymatic activity. DUB immunoprecipitates were examined for DUB activity with Ub-AMC assay (mean $\pm$ SE; $p < 0.05$ for USP1 activity in control versus treated cell lysates, $n=3$). **Immunoblot:** DUB immunoprecipitates were subjected to immunoblotting with antibodies specific against USP1, USP2, USP5, USP7, or USP14.

Figure 3. SJB binds to USP1 and inhibits its deubiquitylating enzymatic activity
(A) MM.1S cells were treated with DMSO or SJB for 3h; protein lysates were incubated with HA-Ub-VS probe for 30 mins, followed by immunoblot analysis using antibodies against USP1, USP2, USP5 or USP7. (B) Ub-chain disassembly reactions of K48-linked Ub tetramers by purified recombinant USP1/UAF1 complex after 30 mins incubation with DMSO, USP1 inhibitor SJB, or proteasome inhibitor marizomib.

Figure 4. Cytotoxic activity of SJB against MM cells

(A) Indicated MM-cell lines were treated with DMSO control or SJB for 24h, followed by assessment for cell viability using WST-1 assay (mean $\pm$ SE; $p < 0.05$ for all cell lines; $n=3$). Cell viability data are presented in a Heatmap. (B) CD138+ patient MM cells were treated with DMSO or SJB for 24h, followed by assessment for cell viability of patient samples (Pt #1-Pt #5) using CellTiter-Glo assay (mean $\pm$ SE; $p < 0.001$, $n=3$). (C) Normal donor PBMCs were treated with SJB for 24h, and then analyzed for viability using WST-1 assay (mean $\pm$ SE of quadruplicate cultures). (D) MM.1S cells were cultured with or without BMSCs for 24h in the presence or absence of SJB, and cell growth was assessed using WST1 assay (mean $\pm$ SE of triplicate cultures; $p < 0.05$ for all samples). (E) MM.1S cells were cultured with or without patient plasmacytoid dendritic cells (pDCs) for 24h with or without SJB, and cell growth was assessed by WST1 assay (mean $\pm$ SE of triplicate cultures; $p < 0.005$ for all samples).

Figure 5. Mechanisms of action of SJB in MM cells

(A) MM.1S cells were treated with SJB for 15h, and analyzed for DNA content with propidium iodide (PI) staining and FACS. Percentage of cell populations in G2/M-, S-, or G1-phase of cell cycle is shown in bar graph (mean $\pm$ SD; $n = 3$; $p < 0.001$). **Immunoblot:** MM.1S cells were treated with indicated concentrations of SJB for 15h; protein lysates were then subjected to immunoblot analysis using anti-p21 and anti- β -actin Abs. (B) **Bar graph:** MM.1S cells were treated with SJB for 15h, and then analyzed for apoptosis using Annexin V/PI staining (mean $\pm$ SD; $n = 3$; $p < 0.005$). **Immunoblot:** MM.1S cells were treated with SJB for 12h; protein lysates were subjected to western blotting using antibodies directed against PARP, caspase-3, caspase-8, caspase-9 or β -actin. (C) MM.1S cells were treated with DMSO control or SJB for 12h; protein lysates were subjected to immunoblot analysis using antibodies specific against with FANCD2, FANCI, PCNA, or GAPDH. (D) MM.1S cells were treated with DMSO or SJB for 6h, and cells were stained with anti-RAD51 Abs. DAPI was used as counterstain for nuclei. **Bar graph:** Quantification of RAD51 Foci/nuclei using multiple fields. (E) MM.1S cells were treated with DMSO or SJB for 4h; cells were washed with plain medium to remove SJB, and then cultured in fresh complete medium for 24h, followed by cell viability cell viability analysis (mean $\pm$ SE; $p < 0.05$, $n=3$). In addition, cells were treated with SJB continuously for 24h, and then subjected to viability analysis (mean $\pm$ SE; $p < 0.05$, $n=3$).