

# 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. Deubiquitylating enzyme USP1 participates in DNA damage response and cellular differentiation pathways. To date, the role of USP1 in multiple myeloma biology is not defined. In the present study, we investigated the functional significance of USP1 in multiple myeloma 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 multiple myeloma cells.

**Results:** USP1-siRNA knockdown decreases multiple myeloma cell viability. USP1 inhibitor SJB selectively blocks USP1 enzymatic activity without blocking other DUBs. SJB also decreases the viability of multiple myeloma cell lines and patient tumor cells, inhibits bone marrow plasmacytoid dendritic cell-induced mul-

tipule myeloma cell growth, and overcomes bortezomib resistance. SJB triggers apoptosis in multiple myeloma 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 multiple myeloma 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 multiple myeloma therapy. *Clin Cancer Res*; 23(15); 4280–9. ©2017 AACR.

## Introduction

Proteasome inhibitors are effective therapy for relapsed/refractory, relapsed, and newly diagnosed multiple myeloma; however, the development of resistance and relapse of disease is common (1–3). 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 multiple myeloma (4–8).

DUBs deconjugate ubiquitin from targeted proteins and facilitate regeneration of free ubiquitin pools (4). In addition, DUBs

maintain cellular protein homeostasis by modulating protein activation, turnover rate, recycling, and localization (4). 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 5 families: the USP (the ubiquitin-specific processing protease), UCH (the ubiquitin C-terminal hydrolase), OTU (the ovarian tumor), MJD (the Josephin domain), and JAMM (the Jab1/Mov34 metalloenzyme). The first 4 families are cysteine proteases, whereas the fifth family is metalloproteases, and to date, USP and UCH are the best characterized families (4).

USP1 regulates DNA repair through Fanconi anemia (FA) pathway by deubiquitylating DNA repair proteins, FANCD2-Ub and PCNA-Ub (9). 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 (HR) pathway (10). Conversely, inhibition of USP1 sensitizes cancer cells to chemotherapy and radiation (9). As USP1 participates in DNA damage response pathways, USP1-knockout mice are genetically unstable and highly sensitive to DNA damage (11). Finally, USP1 inhibits cell differentiation by stabilizing tumor-promoting inhibitor of DNA-binding (ID) proteins (12, 13). To date, the role of USP1 in multiple myeloma biology is undefined. In the present study, we investigated the functional significance of USP1 in multiple myeloma using genetic and biochemical approaches.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

Proteasome inhibitors are effective therapy for multiple myeloma; however, the development of resistance and relapse of disease are 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 multiple myeloma. In this study, we show that USP1 inhibition by SJB3-019A decreases cell viability in multiple myeloma 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-multiple myeloma activity. Our preclinical data support clinical investigation of USP1 inhibitors alone or in combination with other agents in multiple myeloma.

## Materials and Methods

### Cell culture and reagents

Multiple myeloma cell lines and normal donor-derived peripheral blood mononuclear cells (PBMC) were cultured in complete medium containing 10% FBS and antibiotics. All cell lines were tested for mycoplasma contamination using MycoAlert™ mycoplasma detection kit (Lonza). Plasmacytoid dendritic cells (pDC), bone marrow stromal cells (BMSC), or tumor cells from patients with multiple myeloma were purified and cultured as described previously (14). All patient samples were obtained with prior informed consent in accordance Helsinki protocol. Bone marrow mononuclear cells (MNC) were purchased from Allcells (USA). SJB3-019A (SJB), 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 previously (15). Cell viability was assessed by WST-1/CellTiter-Glo (CTG) Luminescent assays, as in prior study (16). Apoptosis was measured by Annexin/propidium iodide (PI) staining.

### Western blotting assays

Immunoblotting was performed as previously described (15) using antibodies against USP1, USP2, USP5, USP7, USP14, caspase-3/8/9, p21, FANCD2, FANCI, PCNA, Rad51, GAPDH (Cell Signaling); ID1, ID2, ID3, ID4, Notch-1, Notch-2, Sox-4, and Sox-2 (Bethyl Laboratories).

### Transfection assays

MM.1S cells were transiently transfected with control scr-siRNA or USP1-siRNA using the Cell Line Nucleofector Kit V (Amaxa Biosystems). Cells were harvested 24 hours posttransfection, followed by analysis using both immunoblotting and cell viability assays.

### Ubiquitin vinyl sulfone labeling, Ub-AMC, and tetraubiquitin chain cleavage assays

Cells were treated with or without SJB for 3 hours and were harvested and lysed. Total protein (25 µg) was labeled with hemagglutinin (HA)-linked Ub-VS probe (1 µmol/L) for 30 minutes at 37°C and analyzed with immunoblotting.

### Ub-AMC assay

recombinant DUBs (rDUB; USP1/UAF1, USP2, USP5, USP7, or UCH37) were incubated with SJB for 30 minutes at 37°C, and then UB-AMC was added for another 30 minutes, followed by measurement of fluorescence intensity.

### Ubiquitin-linked K48 chain cleavage assay

Purified rDUBs were incubated with SJB for 30 minutes, followed by the addition of K48-linked tetraubiquitin chains. The reaction was terminated after 30 minutes by addition of reducing buffer, and samples were analyzed by Western blotting (17).

### Immunostaining

Multiple myeloma cells were stained with Rad51 Ab and Giemsa stain as described previously, and sections were then imaged by microscopy (18).

### USP1 gene expression analysis

The exon-1.0 ST array data for 170 newly diagnosed patients with multiple myeloma 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>.

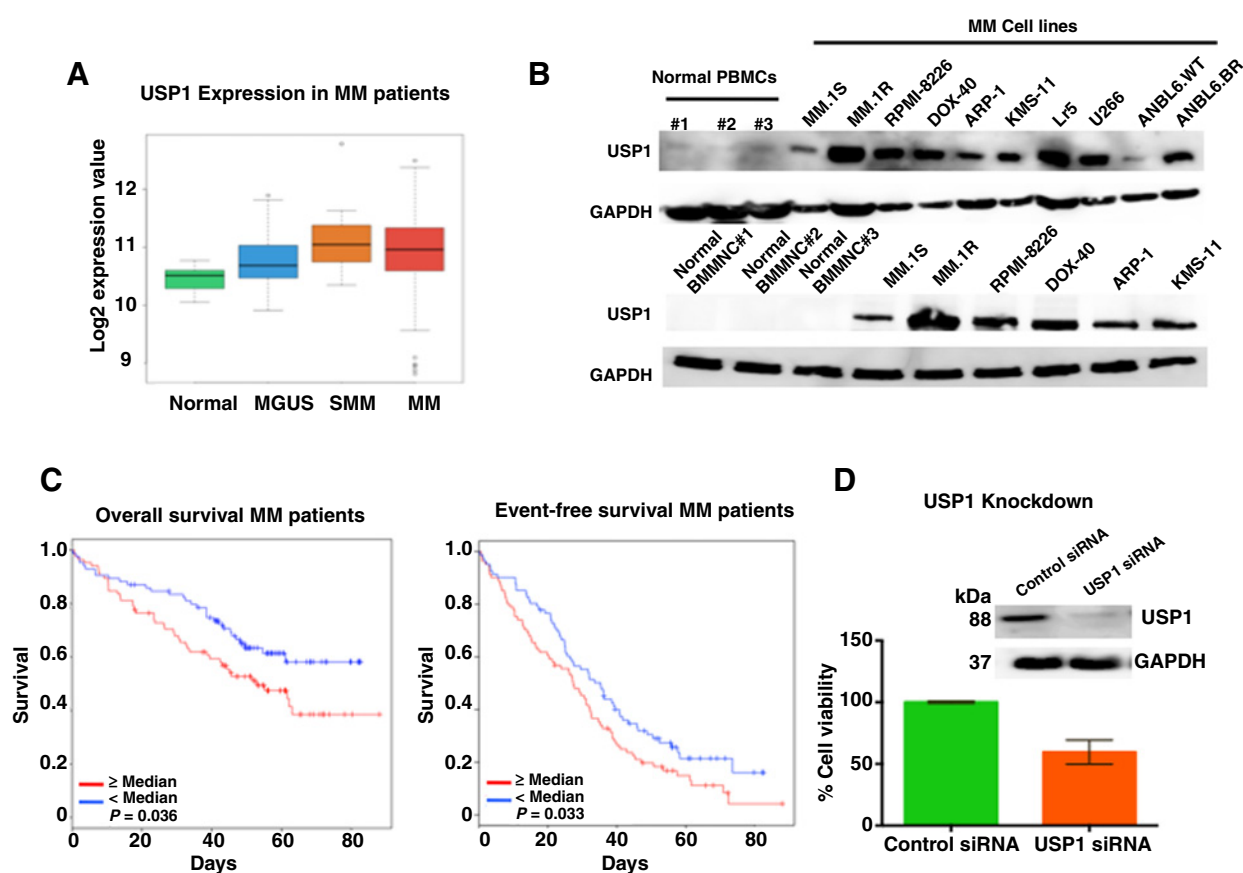
### Statistical analysis

The Student *t* test was utilized to derive statistical significance. Synergistic cytotoxic activity of combination regimes was assessed with isobologram analysis and CalcuSyn software program (19).

## Results

### USP1 expression analysis in multiple myeloma cells

Examination of gene expression datasets showed a higher USP1 in clonal plasma cells from patients with monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), and active multiple myeloma versus normal plasma cells (Fig. 1A). Immunoblot analysis showed elevated USP1 levels in a panel of multiple myeloma cell lines versus normal healthy donor-derived bone marrow MNCs or PBMCs (Fig. 1B). The prognostic relevance of USP1 was assessed by correlating baseline USP1 expression in bone marrow biopsy samples with overall and event-free survival of 170 patients with multiple myeloma. All patients analyzed in this study were newly diagnosed, and no therapy was administered at the time of expression profiling (20). We found a statistically significant inverse correlation between USP1 levels and both overall and



**Figure 1.**

USP1 in multiple myeloma. **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 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 patients with MM. Red line indicates patient group with higher USP1 expression, and blue line shows 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 24 hours, followed by analysis of cell viability by WST assay. The percentage of cell viability was normalized against scr-siRNA control (mean  $\pm$  SE; *P* < 0.005, *n* = 3). Western blot analysis shows USP1 expression in cells transfected with Scr-siRNA or USP1 siRNA.

event-free survival (Fig. 1C). These findings indicate a role of USP1 in multiple myeloma pathogenesis.

To determine the functional significance of USP1 in multiple myeloma, we performed loss-of-function studies with USP1-siRNA. Transfection of USP1-siRNA, but not scr-siRNA, reduces multiple myeloma cell viability (Fig. 1D, bar graph; *P* < 0.05). These data show a role for USP1 in survival of multiple myeloma cells.

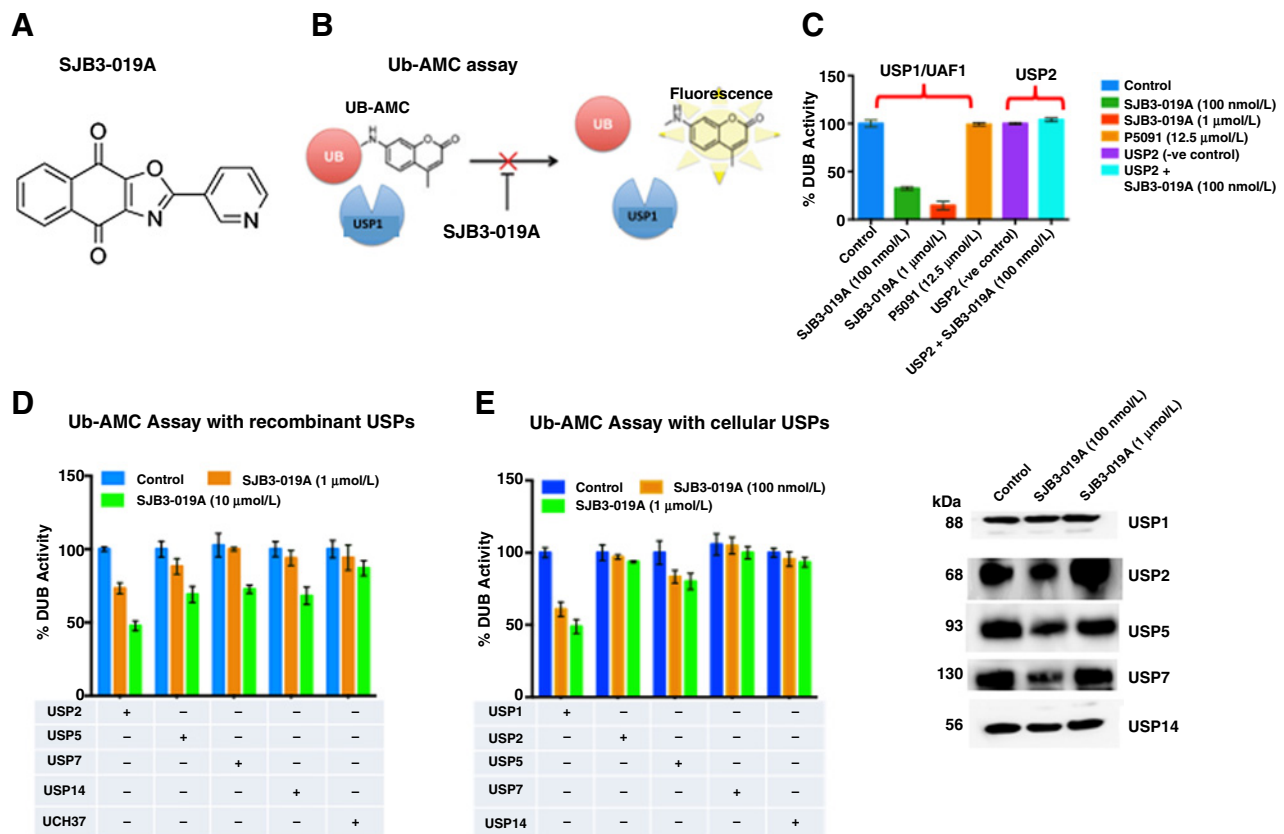
#### Biochemical characterization of USP1 inhibitor SJB

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

used as a positive control for USP1 inhibition assays (Supplementary Fig. S1).

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; Fig. 2E, bar graph). A higher concentration of SJB (1  $\mu$ mol/L) modestly inhibited USP5 activity. Immunoblotting shows that equivalent USP1 immunoprecipitates were analyzed for DUB activity assay (Fig. 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 about 10 kDa (Fig. 3A). Importantly, this conjugate formation was inhibited in the presence of SJB for USP1 enzyme, but not other DUBs. Immunoblotting with anti-HA antibody showed equal protein loading of control and treated protein lysates (Supplementary



**Figure 2.** Biochemical characterization of USP1 inhibitor SJB. **A**, Chemical structure of SJB-019A. **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 minutes at 37°C, followed by the assessment of DUB activity using Ub-AMC assay (mean ± 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 minutes at 37°C, followed by assessment of DUB activity using Ub-AMC assay (mean ± SE;  $P < 0.05$ ,  $n = 3$ ). **E**, MM.1S cells were treated with DMSO control or SJB for 3 hours; 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 ± 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.

Fig. S2). These data suggest that SJB binds to cellular USP1 and blocks its deubiquitylating activity. Furthermore, analysis using K48-linked ubiquitin tetramer chain cleavage assays showed that SJB inhibits USP1-mediated ubiquitin chain disassembly in a concentration-dependent manner (Fig. 3B). Proteasome inhibitor marizomib served as negative control in these assays. No significant inhibitory effect of SJB was noted in USP7- or USP2-mediated ubiquitin chain disassembly (Supplementary Fig. S3). Taken together, these findings provide evidence for the specificity of SJB against USP1.

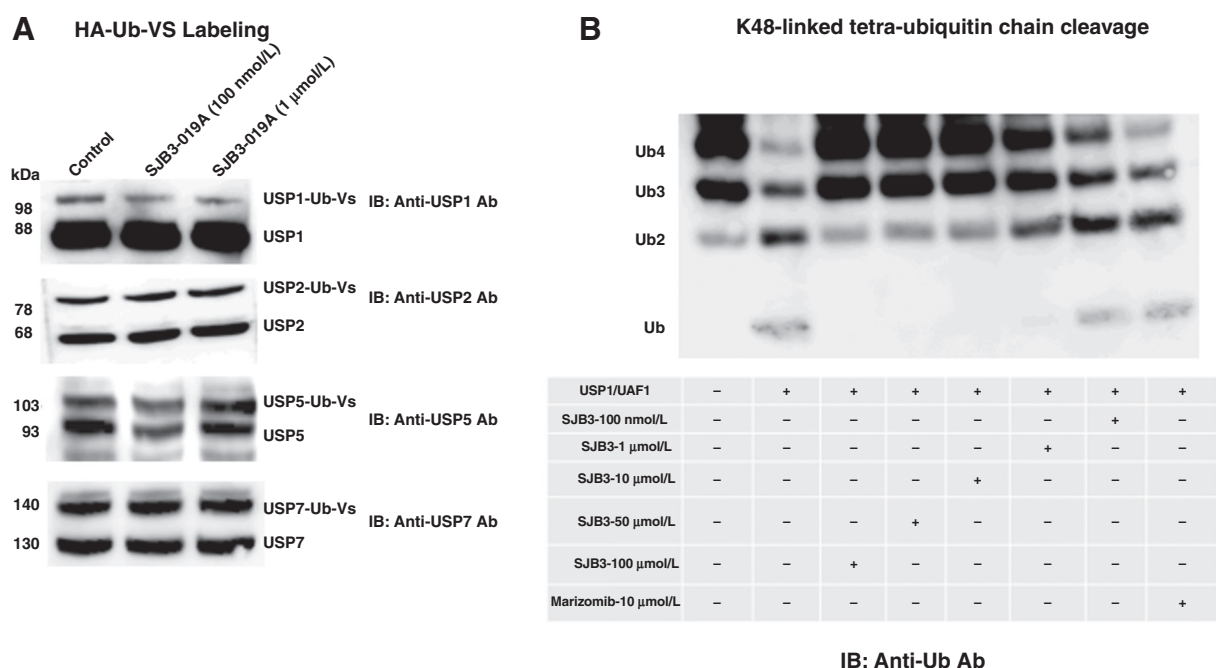
**Cytotoxic activity of SJB against multiple myeloma**

SJB reduces the viability of all multiple myeloma cell lines in a concentration-dependent manner ( $IC_{50}$  ranges from 100 to 500 nmol/L; Fig. 4A). Treatment of purified multiple myeloma cells from patients showed a concentration-dependent decrease in the viability of all patient multiple myeloma cells (Fig. 4B). In contrast, no significant effect of SJB was noted against normal PBMCs (Fig. 4C). However, the highest concentration of SJB (1 μmol/L) decreases viability of PBMCs by 10% to 20%. These

data suggest a favorable therapeutic index for SJB in multiple myeloma. A comparative analysis of SJB with other reported USP1 inhibitors (ML323, SJB2-043, pimozone, GW7647) showed a more potent and selective anti-multiple myeloma activity of SJB (Supplementary Fig. S4).

**SJB activity in the multiple myeloma bone marrow microenvironment**

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

**Figure 3.**

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

### Mechanistic insight into the SJB activity

Cell-cycle analysis showed that SJB induces G<sub>1</sub> phase growth arrest associated with decrease in G<sub>2</sub>-M and S-phases (Fig. 5A). In concert with these findings, SJB upregulated G<sub>1</sub>-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 Fig. S5A).

### SJB disrupts HR in multiple myeloma cells

USP1 regulates DNA repair and FA pathways through its association with binding partner UAF1 (27). In particular, USP1 regulates DNA damage response by deubiquitylation of DNA repair proteins, Ub-FANCD2 and Ub-PCNA (9). We therefore examined whether SJB-mediated inhibition of USP1 affects FA signaling pathway in multiple myeloma cells. MM.1S cells were treated with SJB for 12 hours, and cellular levels of Ub-FANCD2, Ub-FANCI, and Ub-PCNA were then analyzed by Western blotting. As shown in Fig. 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 HR (10), and we next examined the effects of SJB on HR in multiple myeloma cells. For these studies, we assessed whether SJB alters expression of HR-associated RAD51 protein (28). RAD51 accumulates at discrete foci on chromosomal DNA during meiotic prophase; and importantly, DNA damage triggers RAD51 foci formation (28). Our study showed high levels of RAD51 protein in multiple myeloma cells, indicating ongoing DNA damage in these cells (29). Treatment of multiple myeloma cells with SJB

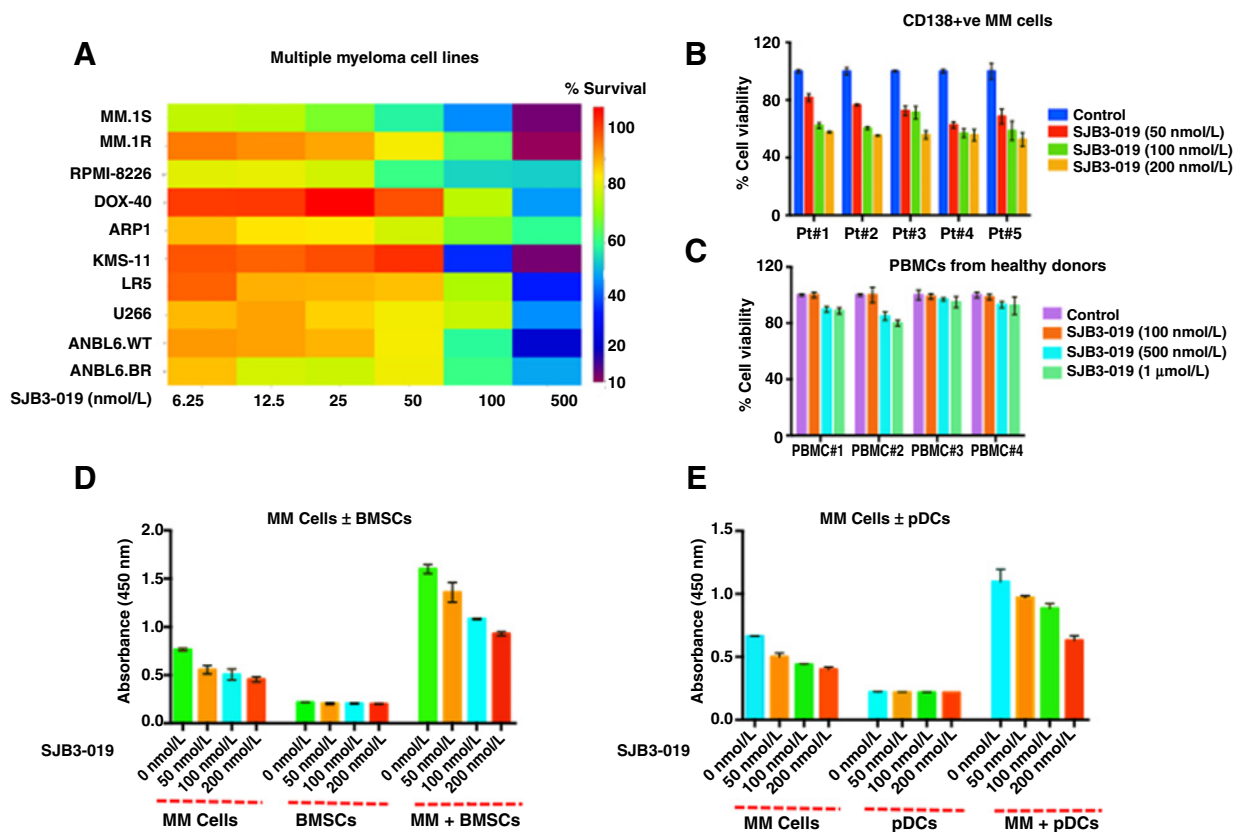
decreased RAD51 foci formation, assessed by immunofluorescent studies (Fig. 5D). Quantification of immunofluorescent data showed decreased RAD51 foci/nuclei in SJB-treated MM.1S cells (Fig. 5D). Together, these findings suggest that SJB abrogates HR mechanisms in multiple myeloma cells.

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 (4 hours); cells were then washed to remove SJB and cultured in complete medium for another 24 hours, followed by analysis of cell viability. Results showed that short-term (4 hours) treatment with SJB triggers significant cytotoxicity in MM.1S cells (Fig. 5E). Although the extent of MM.1S cell death triggered by short-term (4 hours) 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 morphologic differentiation and maturation of multiple myeloma cells by blocking USP1-associated downstream signaling via ID proteins

Inhibitor of ID proteins belongs to a class of helix-loop-helix (HLH) family of transcriptional regulatory proteins, which consists of 4 members (ID1-ID4; ref. 30). ID proteins inhibit bHLH protein-mediated transactivation of genes involved in cellular differentiation pathways (30). Importantly, ID1 is downstream target of USP1 (31). Treatment of MM.1S cells with SJB decreased USP1 and ID1 protein levels (Fig. 6A). Besides ID1, SJB treatment also decreased other ID protein family members ID2, ID3, and ID4 (Fig. 6A). Treatment of resistant cell line Dox-40 slightly reduced USP1 levels and did not affect





**Figure 4.**

Cytotoxic activity of SJB against MM cells. **A**, Indicated MM cell lines were treated with DMSO control or SJB for 24 hours, 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<sup>+</sup> patient MM cells were treated with DMSO or SJB for 24 hours 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 24 hours 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 24 hours in the presence or absence of SJB, and cell growth was assessed using WST-1 assay (mean  $\pm$  SE of triplicate cultures;  $P < 0.05$  for all samples). **E**, MM.1S cells were cultured with or without patient pDCs for 24 hours with or without SJB, and cell growth was assessed by WST-1 assay (mean  $\pm$  SE of triplicate cultures;  $P < 0.005$  for all samples).

ID protein levels (Supplementary Fig. S5B). Importantly, GEP analysis of ID1 and ID2 proteins showed an inverse correlation with survival in patients with multiple myeloma (Supplementary Fig. S6).

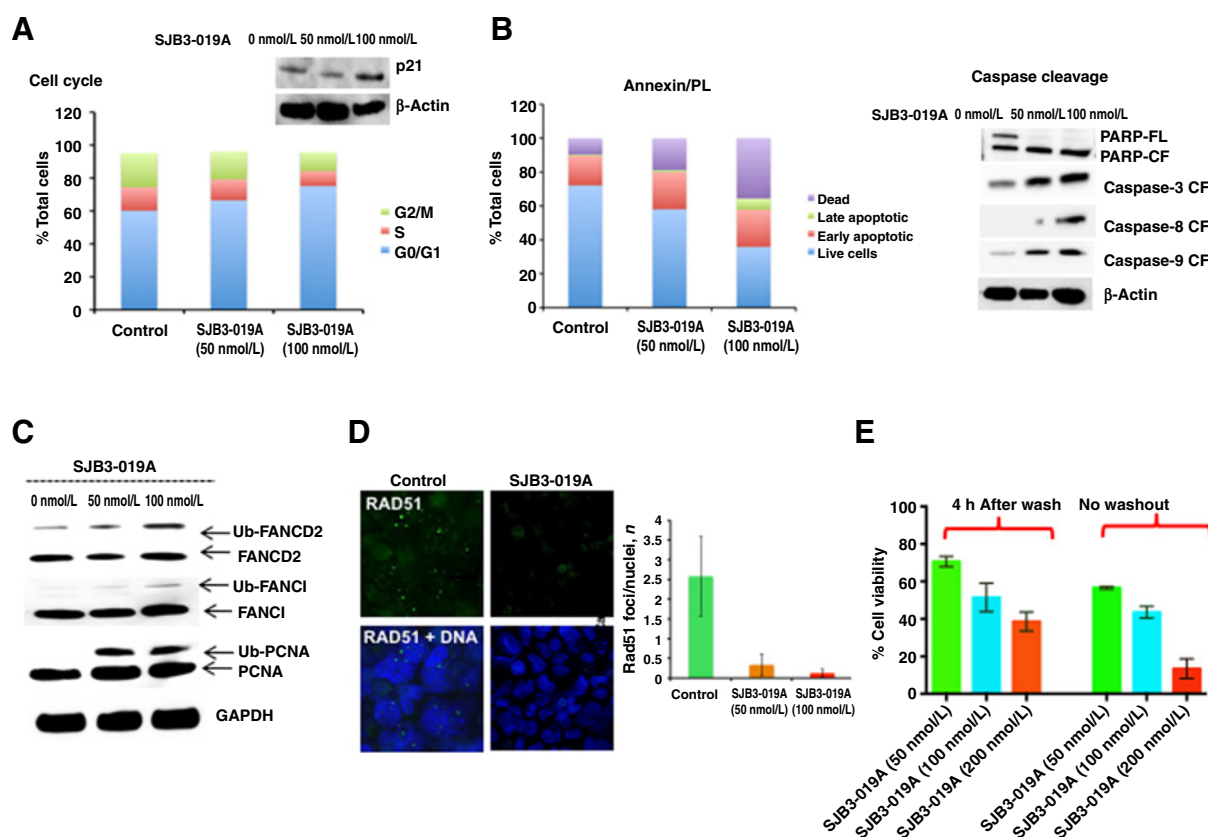
Earlier studies showed that ID proteins are highly expressed in various cancer types including multiple myeloma and promote stem cell survival (30, 32). As SJB downregulates ID proteins, we examined whether SJB affects differentiation of multiple myeloma cells using Giemsa staining assays. A significantly decreased nuclear-cytoplasmic ratio was observed in SJB- versus DMSO/control-treated MM.1S cells (Fig. 6B). SJB-treated cells showed a more mature and differentiated state versus untreated cells: immature plasmablasts with large nuclei in untreated cells (Fig. 6B, left) versus condensed nuclei with rough chromatin pattern and a more prominent cytoplasm in SJB-treated cells (Fig. 6B, right). These data indicate that SJB induces plasma cell differentiation with prominent cytoplasm and a reduced nuclear-cytoplasmic ratio.

Previous studies showed that ID proteins contribute to chemoresistance and cancer stemness (32). For example, ID4 regulates the expression of stemness-associated Sox2 and Notch signaling in glioma cells (33, 34). We found that

SJB-mediated inhibition of USP1/ID signaling led to down-regulation of stemness-associated Sox-4/Sox-2/Notch-1/Notch-2 signaling proteins in multiple myeloma cells (Fig. 6C). In addition, Notch signaling promotes the maintenance and proliferation of hematopoietic stem cells (35). Previous studies have reported presence of multiple myeloma clonal side population (MM-SP) with characteristic stem-like features (36). We therefore here evaluated the effect of SJB on MM-SP using flow cytometric analysis of Hoechst 33342-stained cells. SJB significantly (38% decrease) lowered the fraction of MM-SP (Fig. 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-multiple myeloma agents (37). Isobologram analysis for synergistic anti-multiple myeloma activity showed that the combination of SJB and bortezomib triggers synergistic anti-multiple myeloma activity

**Figure 5.**

Mechanisms of action of SJB in MM cells. **A**, MM.1S cells were treated with SJB for 15 hours and analyzed for DNA content with PI staining and FACS. Percentage of cell populations in G<sub>2</sub>-M, S, or G<sub>1</sub> phase of cell cycle is shown in bar graph (mean ± SD; *n* = 3; *P* < 0.001). Immunoblot: MM.1S cells were treated with indicated concentrations of SJB for 15 hours; protein lysates were then subjected to immunoblot analysis using anti-p21 and anti-β-actin antibodies. **B**, Bar graph: MM.1S cells were treated with SJB for 15 hours and then analyzed for apoptosis using Annexin V/PI staining (mean ± SD; *n* = 3; *P* < 0.005). Immunoblot: MM.1S cells were treated with SJB for 12 hours; 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 12 hours; protein lysates were subjected to immunoblot analysis using antibodies specific against FANCD2, FANCI, PCNA, or GAPDH. **D**, MM.1S cells were treated with DMSO or SJB for 6 hours, and cells were stained with anti-RAD51 antibodies. 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 4 hours; cells were washed with plain medium to remove SJB and then cultured in fresh complete medium for 24 hours, followed by cell viability analysis (mean ± SE; *P* < 0.05, *n* = 3). In addition, cells were treated with SJB continuously for 24 hours and then subjected to viability analysis (mean ± SE; *P* < 0.05, *n* = 3).

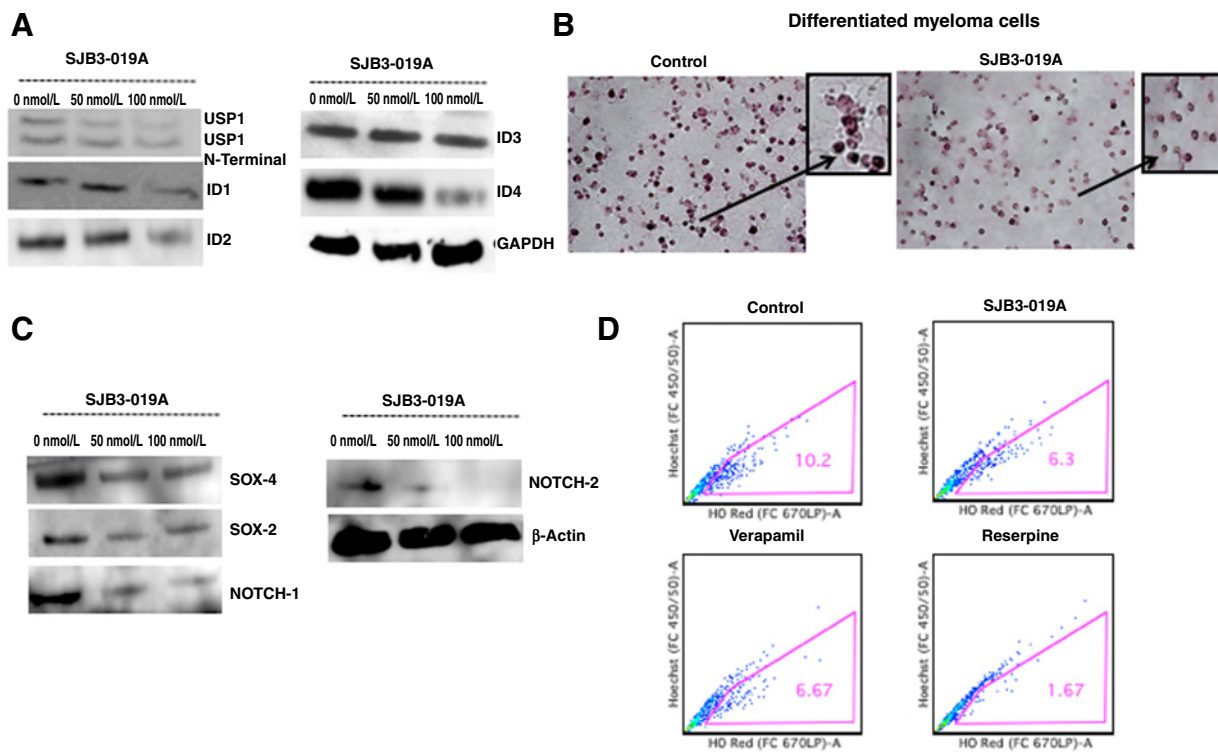
(Supplementary Fig. S7A). The mechanism of synergy between SJB and bortezomib may include enhanced accumulation of ubiquitinated proteins, which are not degraded and triggering of distinct cell death signaling pathways.

Protein degradation also utilizes HDAC-dependent aggressive autophagic cell death signaling pathway. Interestingly, the combination of SJB and HDAC6 inhibitor ACY-1215 triggers synergistic anti-multiple myeloma cytotoxicity (Supplementary Fig. S7B). Similarly, we found that combination of SJB and lenalidomide (Supplementary Fig. S7C) or pomalidomide (Supplementary Fig. S7D) triggered synergistic cytotoxicity against multiple myeloma cells. These combination studies were also performed using primary multiple myeloma tumor cells (Supplementary Fig. S8). These data provide the framework for scientifically informed clinical trials combining strategies to inhibit USP1 with standard-of-care anti-multiple myeloma agents.

## Discussion

We utilized multiple myeloma cell lines, patient cells, and co-culture models of multiple myeloma cells with BMSCs or pDCs, as well as genetic and biochemical and strategies, to validate DUB enzyme USP1 as a therapeutic target in multiple myeloma. USP1 is highly expressed in multiple myeloma cells compared with 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 patients with multiple myeloma.

RNA interference and biochemical strategies were utilized to determine the functional significance of USP1 in multiple myeloma. USP1-siRNA decreases multiple myeloma cell viability. We utilized a pharmacologic inhibitor of USP1 SJB that targets USP1 (30). The specificity of SJB was confirmed by various experiments: (i) we show that SJB potently and selectively blocks USP1 activity



**Figure 6.**

SJB degrades USP1 and ID proteins and alters MM cell morphology. **A**, MM.1S cells were treated with DMSO control or SJB for 12 hours; protein lysates were subjected to immunoblot analysis using antibodies directed against USP1, ID1, ID2, ID3, ID4, or GAPDH. **B**, MM.1S cells were treated with DMSO control or SJB for 6 hours; cells were seeded on the glass slides and stained with Giemsa stain. **C**, MM.1S cells were treated with DMSO control or SJB for 12 hours; protein lysates were subjected to immunoblot analysis using antibodies directed against SOX-4, SOX-2, NOTCH-2, NOTCH-1, or  $\beta$ -actin. **D**, RPMI-8226 cells were treated with DMSO control or SJB for 12 hours and subjected to dot plot analysis of MM-SP using FACS. MM-SP was sorted using staining and FACS. Hoechst-33342 accumulation was assessed in RPMI-8226 cells cultured with DMSO alone or in the presence of SJB (100 nmol/L), verapamil (100  $\mu$ mol/L), and reserpine (50  $\mu$ mol/L). Abscissa represents Hoechst red fluorescence intensity, and ordinate is Hoechst blue fluorescence intensity, with flow gate representing the MM-SP fraction of RPMI-8226 cells.

without inhibiting other DUBs (USP2/USP5/USP7/USP14/UCH37); (ii) SJB inhibited binding of USP1 with HA-Ub-V5 probe, but it did not affect labeling of other DUBs with probe; and (iii) 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 cells (30). Here, we show that SJB triggers apoptosis in multiple myeloma cells *in vitro*, without reducing the viability of normal PBMCs. Moreover, SJB triggered more potent anti-multiple myeloma activity versus other available USP1 inhibitors (ML323, SJB2-043, pimozide, GW7647; ref. 31). Of note, we found that SJB retains its activity against multiple myeloma cell lines resistant to conventional and novel therapies. Distinct genetic backgrounds (38) and/or drug resistance characteristics may account for the differences in  $IC_{50}$  of SJB against multiple myeloma cell lines. Differential USP1 expression was observed among various multiple myeloma 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 multiple myeloma cell lines. Our data in multiple myeloma model are consistent with cytotoxic effects of USP1 inhibition observed in leukemic cells (31).

We next assessed whether SJB can overcome bortezomib resistance in multiple myeloma cells. For these studies, we utilized bortezomib-sensitive (ANBL6.WT) and -resistant (ANBL6.BR) multiple myeloma cell lines (39). SJB induced cytotoxicity in ANBL6.BR cells, which confirmed the ability of SJB to overcome bortezomib resistance. In addition, SJB was active against tumor cells from patients with multiple myeloma resistant to novel (bortezomib, lenalidomide), conventional (dexamethasone) agents. The bone marrow microenvironment (BMSCs and pDCs) promotes multiple myeloma cell growth, survival, and drug resistance; importantly, SJB triggers apoptosis in multiple myeloma cells even in the presence of the bone marrow milieu.

USP1-UAF1 regulates DNA damage response signaling pathways (9). We examined the effect of SJB on the 3 essential DNA repair pathways associated with USP1: (i) FA pathway (via FANCD2/FANCI), (ii) translesion synthesis (via PCNA), and (iii) DNA double-strand break repair through HR (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 pathway (31). We found that USP1 inhibition by SJB increased the levels of both Ub-FANCD2 and Ub-FANCI in multiple myeloma cells. These findings suggest that SJB inhibits the FA repair pathway in multiple myeloma cells and that SJB may sensitize multiple myeloma



cells to DNA-damaging anti-multiple myeloma 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). In addition, USP1 plays a role in HR-mediated DNA repair, and USP1 blockade disrupts this pathway. Here, we found that multiple myeloma cells express high levels of HR-associated DNA repair protein RAD51. Treatment of multiple myeloma 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 (4 hours) of multiple myeloma cells triggers significant cytotoxicity (Fig. 5E). Together, the ability of SJB to block multiple DNA repair mechanisms likely contributes to its overall potent anti-multiple myeloma activity.

Besides DNA repair proteins, USP1 also promotes stabilization of ID (ID1-ID4) proteins (31). ID proteins are highly expressed in proliferating cells and promote stem cell-like characteristics in osteosarcoma cells (9, 12). Studies in multiple myeloma have shown that histone methyltransferase protein MMESET promotes oncogenic transformation by transcriptional activation of ID1 protein expression (41). In our study, we found that SJB down-regulates ID1, ID2, ID3, and ID4 proteins in multiple myeloma cells, suggesting that it may affect the differentiation state of multiple myeloma cells and/or multiple myeloma stem cell-like cell populations. A prior study showed that subpopulations of multiple myeloma with distinct morphology and immunophenotype are consistent with distinct phases of differentiation (42). In addition, the presence of immature multiple myeloma cells portends poor prognosis, and several studies have reported the survival advantage for patients with plasmacytic versus plasmablastic type multiple myeloma (42-44). In our study, we found that SJB induced multiple myeloma 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 multiple myeloma.

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 identity (30). 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 gliomas (46); and overexpression of ID4 induces activation of Notch signaling and stem cells in gliomas (34). In our study, we showed that SJB inhibits SOX and Notch signaling in multiple myeloma cells. Notch1 and Notch2 are highly expressed in primary multiple myeloma cells and their expression increases with progression from MGUS to multiple myeloma

(35). Moreover, overexpression of Notch receptors/ligands is integral to multiple myeloma stem cell self-renewal and proliferation (35). Finally, SOX2 expression is a key feature of clonogenic multiple myeloma cells (47, 48). Importantly, we here found that inhibition of USP1/ID/Notch/Sox2 pathway by SJB decreased the percentage of clonogenic MM-SP. Taken together, our data show that: (i) SJB triggers immature plasma cells to differentiate, mature, and undergo apoptosis and (ii) SJB decreases multiple myeloma stem cell-like clonogenic population (MM-SP).

Finally, we also examined whether SJB enhances the anti-multiple myeloma activity of other agents. Isobologram analysis for synergistic anti-multiple myeloma activity demonstrated that the combination of SJB with bortezomib, HDAC6i ACY1215, lenalidomide, or pomalidomide triggers synergistic anti-multiple myeloma activity. The mechanism of synergy between SJB and other anti-multiple myeloma 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 multiple myeloma.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

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Development of methodology: D.S. Das, A. Das

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.S. Das, A. Das, A. Ray, Y. Song, N.C. Munshi, K.C. Anderson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.S. Das, A. Das, M.K. Samur, K.C. Anderson

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.S. Das, A. Ray, Y. Song, K.C. Anderson

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