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Synergistic Cytotoxic Effect of Busulfan and the PARP Inhibitor Veliparib in Myeloproliferative Neoplasms

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Patients with high-risk myeloproliferative neoplasms (MPNs), and in particular myelofibrosis (MF), can be cured only with allogeneic hematopoietic stem cell transplantation (HSCT). Because MPNs and JAK2^{V617F}-mutated cells show genomic instability, stalled replication forks, and baseline DNA double-strand breaks, DNA repair inhibition with poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors represents a potential novel therapy. Because the alkylating agent busulfan is integral in conditioning regimens for HSCT and leads to stalled replication forks through DNA strand cross-linking, we hypothesized that PARP inhibition with veliparib in combination with busulfan may lead to synergistic cytotoxicity in MPN cells. We first treated 2 MPN cell lines harboring the JAK2^{V617F} mutation (SET2 and HEL) with veliparib at increasing concentrations and measured cell proliferation. SET2 and HEL cells were relatively sensitive to veliparib (IC_{50} of 11.3 μM and 74.2 μM, respectively). We next treated cells with increasing doses of busulfan in combination with 4 μM veliparib and found that the busulfan IC_{50} decreased from 27 μM to 4 μM in SET2 cells and from 45.1 μM to 28.1 μM in HEL cells. The mean combination index was .55 for SET2 cells and .40 for HEL cells. Combination treatment of SET2 cells caused G2M arrest in 53% of cells, compared with 30% with veliparib alone and 35% with busulfan alone. G2M arrest was associated with activation of the ATR-Chk1 pathway, as shown by an immunofluorescence assay for phosphorylated Chk1 (p-Chk1). We then tested in vivo the effect of combined low doses of busulfan and veliparib in a JAK2^{V617F} MPN-AML xenotransplant model. Vehicle- and veliparib-treated mice had similar median survival of 39 and 40 days, respectively. Combination treatment increased median survival from 47 days (busulfan alone) to 50 days (P = .02). Finally, we tested the combined effect of busulfan and veliparib on CD34^{+} cells obtained from the bone marrow or peripheral blood of 5 patients with JAK2^{V617F}-mutated and 2 patients with CALR-mutated MF. MF cells treated with the combination of veliparib and busulfan showed reduced colony formation compared with busulfan alone (87% versus 68%; P = .001). In contrast, treatment of normal CD34^{+} cells with veliparib did not affect colony growth. Here we show that in vivo confirmation that treatment with the PARP-1 inhibitor veliparib and busulfan results in synergistic cytotoxicity in MPN cells. Our data provide the rationale for testing novel pretransplantation conditioning regimens with combinations of PARP-1 inhibition and reduced doses of alkylators, such as busulfan and melphalan, for high-risk MPNs or MPN-derived acute myelogenous leukemia.

INTRODUCTION

Despite the discovery of gene mutations (eg, *JAK2, CALR, MPL*) associated with myeloproliferative neoplasms (MPN) and the development of new targeted therapeutics [1], patients with high-risk MPNs, in particular myelofibrosis (MF), have an increased chance of developing secondary acute myelogenous leukemia (AML) and can be cured only with allogeneic hematopoietic stem cell transplantation (HSCT) [2,3]. Furthermore, AML secondary to MPN (MPN-AML) is often refractory to standard chemotherapy and carries a high risk of relapse even with HSCT [4]. The development of therapies prolonging survival of patients with MPNs is an urgent unmet need.

Genomic instability of MPNs and *JAK2*^{V617F}-mutated cells has been previously demonstrated, with baseline DNA double-strand breaks and homologous recombination (HR) activity as a result of stalled replication forks [5-7]. The DNA damage-sensing enzyme poly(ADP-ribose) polymerase-1 (PARP-1) is

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central to detecting these disrupted replication forks and recruiting recombination repair enzymes. Because the alkylating agent busulfan is integral to conditioning regimens for HSCT in MF and MPN-AML and also leads to stalled replication forks through DNA strand cross-linking, we hypothesized that PARP inhibition with veliparib (ABT-888) in combination with busulfan may lead to synergistic cytotoxicity in MPN cells.

METHODS

Cells and Drugs

Cells from two *JAK2*^{V617F} mutant (SET2 and HEL) and 2 *JAK2* wild-type (HL60 and K562) cell lines (American Type Culture Collection, Manassas, VA) were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in the recommended media with appropriate concentrations of fetal bovine serum (Mediatech, Manassas, VA) and 1% penicillin/ streptomycin (Lonza, Walkersville, MD). Cells were maintained for a maximum of 20 passages to minimize long-term culturing effects. Busulfan (Sigma-Aldrich, St Louis, MO) was freshly dissolved in DMSO immediately before cell treatment. Veliparib (Santa Cruz Biotechnology, Dallas, TX) was maintained dissolved in DMSO. The final concentration of DMSO diluted in culture medium was ≤.5% for all experiments.

Proliferative Assay

For single drug treatment, cells were cultured at density of $.1 \times 10^6$ cells/mL and treated with varying concentrations of either busulfan or veliparib. For treatment with multiple drugs, cells were instead treated with veliparib (4 μM) at fixed concentrations and with varying concentrations of busulfan. Following the addition of drugs to liquid culture, 20,000 cells per well were plated in 96-well plates. After 28 hours, cells were pulsed with 1 μCi/well (.037 MBq/well) of 3H-thymidine for 20 hours. After a total of 48 hours, cells were harvested, and mean CPM per well was obtained using a TopCount NXT scintillation counter (Packard, Meriden, CT). The percent inhibition (PI) for each dose was calculated by the formula: PI = mean CPM of treated cells/mean CPM of untreated cells.

Primary Cells

MF CD34$^+$ cells were obtained from peripheral blood or bone marrow of patients. Normal CD34$^+$ cells used as healthy controls were obtained from apheresis collection or cord blood (AllCells, Alameda, CA). Mononuclear cells were subsequently obtained by centrifugation over Ficoll/Hypaque (Amersham Biosciences, Piscataway, NJ) gradients. Mononuclear cells were then washed twice in phosphate buffered-saline (PBS, Cambrex, Walkersville, MD) with 1% BSA (Sigma-Aldrich, St Louis, MO) and CD34$^+$ cells were purified in a MidiMACS high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). To assess purity, aliquots of isolated CD34$^+$ cells were restained with an FITC-conjugated anti-CD34 monoclonal antibody (BD, Franklin Lakes, NJ). Procedures were approved by the University of Illinois Institutional Review Board.

Clonogenic Assay

For assessment of hematopoietic colonies, cells were plated at densities ranging from 0.4×10^3 to 1×10^5 cells/plate in duplicate cultures containing 1 mL of Iscove's Modified Dulbecco's Medium with 1% methylcellulose, 30% FBS, 2 mM L-glutamine, 10^{-4} M 2-mercaptoethanol, and the following cytokines: 3 U/mL rh-erythropoietin (rh-Epo), 50 ng/mL rh stem cell factor (rh-SCF), 20 ng/mL rh granulocyte-macrophage colony-stimulating factor (rh-GM-CSF), 20 ng/mL rh-IL3, 20 ng/mL rh- IL6, and rh-granulocyte colony-stimulating factor (rh-G-CSF) (Methocult GF H4435; Stem Cell Technologies, Vancouver, BC, Canada). Colony-forming cells (CFU-C), including granulocyte-macrophages CFU (CFU-GM), erythroid progenitors (burst-forming unit-erythroid), and CFU-mix, were scored after 14 days of incubation in 35-mm tissue culture dishes at 37°C in a fully humidified 5% CO_2 atmosphere.

Cell Cycle

Cell cycle analysis was performed by PI staining. Cells were pelleted and then fixed overnight in 70% cold ethanol. Cells were then washed, treated with 20 mg/mL RNAse A (Life Technologies, Carlsbad, CA), and stained with PI. Cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA) with cells in different phases determined using CellQuest software.

Immunofluoresence Microscopy

After treatment, cells were washed once in PBS before cytocentrifugation onto polylysine-coated microscope slides. Cells were fixed on slides for 20 minutes in 4% formaldehyde at room temperature followed by cold (-20°C) acetone treatment for 3 minutes. The cells were blocked with 5% normal goat serum (Santa Cruz Biotechnology) in PBS for 30 minutes, followed by incubation with either Alexa Fluor 488 anti-human γ-H2AX (BD Biosciences) or anti-human p-Chk1(S346) and secondary antibody (Cell Signaling Technology, Danvers, MA). Cells were then stained with DAPI for 1 minute. The slides were mounted with prolonged antifading media (Molecular Probes, Eugene, OR) and the proteins were visualized using an Axioskop 2 (Carl Zeiss MicroImaging, Jena, Germany) equipped with a 40× objective (Zeiss Ph2, Plan-Neofluar, 40×/0.75, ∞/0.17).

NSG Xenograft Model of JAK2 *Mutant MPN*

Immunodeficient nonobese diabetic/*ltsz-scid*/*scid* (NOD/SCID) IL-2 receptor gamma chain knockout (NSG) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). SET2 cells (5×10^6) were injected into sublethally irradiated (300 cGy) NSG mice. Vehicle or drug treatment was started at 2 weeks after transplantation. Veliparib was diluted in sterile normal saline, and busulfan was given in DMSO. Intraperitoneal (i.p.) injections were performed 5 days a week for veliparib (3 mg/kg). Busulfan was injected i.p. once weekly at a dose of 25 mg/kg. When mice developed hind limb paralysis, they were sacrificed. Femurs were flushed with RPMI 1640 with 2% FBS and 0.02% sodium azide. A single-cell suspension of spleen cells was prepared by mechanical homogenization of spleen, filtered through a 40-μm cell strainer, followed by depletion of erythrocytes using red blood cell lysing buffer (Sigma-Aldrich). Cells were counted and preincubated with 1 mg/mL human gamma globulin (Bayer, Elkhart, IN) to block human Fc receptors. Murine Fc receptors were blocked by a second incubation of the cells in an anti-mouse Fc receptor monoclonal antibody (2.4G2; BD Pharmingen, San Diego, CA). Marrow and spleen cells were analyzed by flow cytometry to detect engraftment of human CD33$^+$ and CD34$^+$ cells.

Statistical Analysis

The *t* test or ANOVA was performed to compare 2 or more series of data, respectively. Growth inhibition of 50% (GI_{50}) calculation for cell lines, fitting of sigmoidal dose response curves, and survival analysis were done using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Calculation of the CI and dose reduction indexI) for busulfan was performed using Compusyn (Combosyn, Paramus, NJ). CI is a mathematical calculation of synergy (CI <1 indicating synergy; CI ≈ indicating additive; CI >1 suggesting antagonism).

RESULTS

PARP Inhibition with Veliparib Leads to Synergistic Reduction in Proliferation with Busulfan in MPN cells

To evaluate the effect of PARP-1 inhibition on MPNs, we first treated 2 MPN cell lines harboring the *JAK2*^{V617F} mutation (SET2 and HEL) with veliparib at increasing concentrations in liquid cultures and measured cell proliferation by a 3[H]-thymidine uptake assay. We compared these with control *JAK2* wild-type AML cell lines (HL60 and K562). SET2 and HEL cells were relatively more sensitive to veliparib (IC_{50} of 11.3 μM and 74.2 μM, respectively) compared with *JAK2* wild-type cells, which were resistant (Figure 1A). Using an immunofluorescence assay for γH2AX, we showed that cytotoxicity was a result of double-strand DNA breaks in SET2 cells (Figure 1B). Because busulfan is the alkylating agent most commonly used in HSCT conditioning regimens for MPNs, we treated all cell lines with increasing doses of busulfan in combination with 4 μM veliparib for 48 hours. With combination treatment, the busulfan IC_{50} decreased from 27 μM to 4 μM in SET2 cells and from 45.1 μM to 28.1 μM in HEL cells (Figure 1C). The combination was synergistic with a mean CI of 0.55 for SET2 and 0.40 for HEL cells. In contrast, the CI was 0.74 in K562 cells and 0.65 in HL60 cells.

Treatment with Veliparib Induces G2M Arrest Through Chk-1 Activation

As the *JAK2*^{V617F} mutation has been shown to induce replicative stress and lead to constitutive activation of Chk1, we tested whether treatment of SET2 cells with combined veliparib and busulfan would lead to increased Chk1 activation and cell cycle arrest. We first tested for cell cycle arrest and found greater G2M arrest in 53% of combination-treated cells, compared with 30% in cells treated with veliparib alone and 35% in cells treated with busulfan alone (Figure 1D; $P = .002$). Subsequently, we performed an immunofluorescence assay for phosphorylated Chk1 (p-Chk1) to test whether activation of

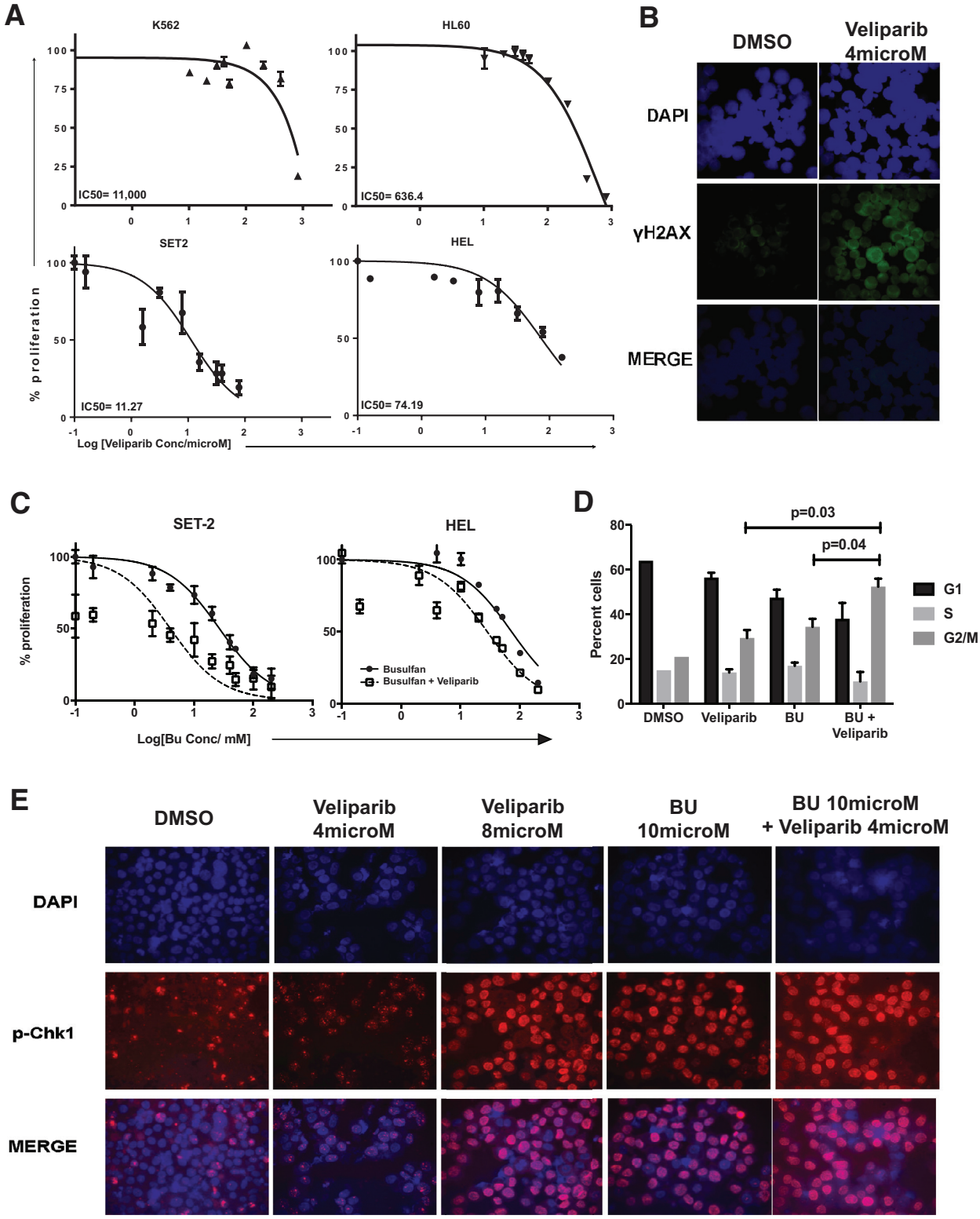

Figure 1. (A) Dose-response curves showing the cytotoxic effect of the PARP inhibitor veliparib on *JAK2* wild-type (*K562* and HL60) and *JAK2* mutant (SET2 and HEL) AML cells. Cells were treated in suspension culture with escalating doses of veliparib, and proliferation was measured after 48 hours with 3[H]-thymidine uptake. JAK^{V617F} cells are relatively sensitive to veliparib, as shown by a markedly lower IC_{50}. (B) Phosphorylated H2AX (γH2AX) staining and visualization by immunofluorescence after 24 hours of treatment of SET2 cells with 4 μM veliparib shows increased staining compared with DMSO-treated control and thus evidence of double-strand DNA damage. (C) Dose-response curves to show the synergistic reduction in proliferation of veliparib with the alkylating agent busulfan. *JAK2* mutant cells were treated with increasing doses of busulfan and a fixed dose (8 μM) of veliparib. Proliferation was measured after 48 hours. The busulfan IC_{50} decreased from 27 μM to 4 μM in SET2 cells and from 45.1 μM to 28.1 μM in HEL cells. (D) G2M arrest in SET2 cells after treatment as shown by PI staining. Cells treated with veliparib, busulfan, or a combination all showed an increase in cells in G2M arrest compared with untreated cells. In addition, combination-treated cells showed an increase in G2M arrest compared with veliparib or busulfan alone (53% versus 30% or 35%). (E) p-Chk-1 staining and visualization by immunofluorescence after 24 hours of treatment in SET2 cells showing dose-dependent activation after veliparib treatment. In addition, combined treatment with veliparib and busulfan led to increased activation compared with either drug alone.

the ATR-Chk1 pathway is associated with G2M arrest. Baseline SET2 cells showed p-Chk1; however, there was a dose-dependent increase in p-Chk1 with veliparib treatment. Busulfan-treated cells showed a further increase, but cells treated with a combination of veliparib and busulfan showed the greatest increase in Chk1 activation (Figure 1E).

The Combination of Busulfan and Veliparib Shows Activity in a JAK2^{V617F} MPN-AML Xenotransplant Model

As proof of principle of the synergistic cytotoxic effect of PARP inhibition with busulfan in MPNs, the in vivo effect of combined low doses of busulfan and veliparib was tested in a *JAK2^{V617F}* MPN-AML xenotransplant model. To establish disease, 5×10^6 SET2 cells were injected via tail vein into NOD/SCID/IL-2Rγ^{null} (NSG) mice after sublethal irradiation. To assess the synergy between veliparib and busulfan, the veliparib dose used was lower than the reported effective dose in mice (usually 5 to 25 mg i.p. twice daily). Drug treatment was started 14 days after SET-2 injection for a total of 3 weeks or less in mice that died earlier (Figure 2A). Mice (n = 5 per group) were treated with vehicle, with veliparib alone (3 mg/kg) for 5 days a week, once-weekly busulfan alone (25 mg/kg), or a combination of both drugs. Death from SET-2 was verified in mice by staining marrow and spleen cells with non-cross-reacting anti-human CD33 and CD34 antibodies (data not shown) and analyzing by flow cytometry. Vehicle- and veliparib-treated mice had similar median survival of 39 and 40 days, respectively. There was a significant difference between curves ($P < .0001$). Specifically, combination treatment increased median survival from 47 days (busulfan alone) to 50 days ($P = .02$; Figure 2B).

Veliparib and Busulfan Exhibit Significant Combined Cytotoxicity in Primary MF Cells but Not in Normal CD34$^+$ Cells

Finally, we tested the effect of PARP inhibition on CD34$^+$ cells obtained from the bone marrow or peripheral blood of 5 patients with *JAK2^{V617F}*-mutated and 2 patients with *CALR*-mutated MF. As a control, we used normal CD34$^+$ cells obtained from healthy donors (AllCells, Alameda, CA). In a standard clonogenic assay in methylcellulose, cells were plated either without drugs or with veliparib at 4 μM, busulfan at 5 μM, or a combination of the 2 drugs. Compared with untreated cells, cells treated with veliparib had a 40% reduction in colony formation ($P = .0001$; Figure 3). In addition, the combination of veliparib and busulfan was associated with reduced colony formation compared with busulfan alone (87% versus 68%; $P = .001$). This effect was seen in cells obtained from patients with *JAK2*- or *CALR*-mutated MF. In contrast, treatment of normal CD34$^+$ cells with veliparib did not affect colony growth. Furthermore, when normal CD34$^+$ cells were treated with both veliparib and busulfan, there was no additional decrease in CFU-C compared with busulfan alone at the doses used.

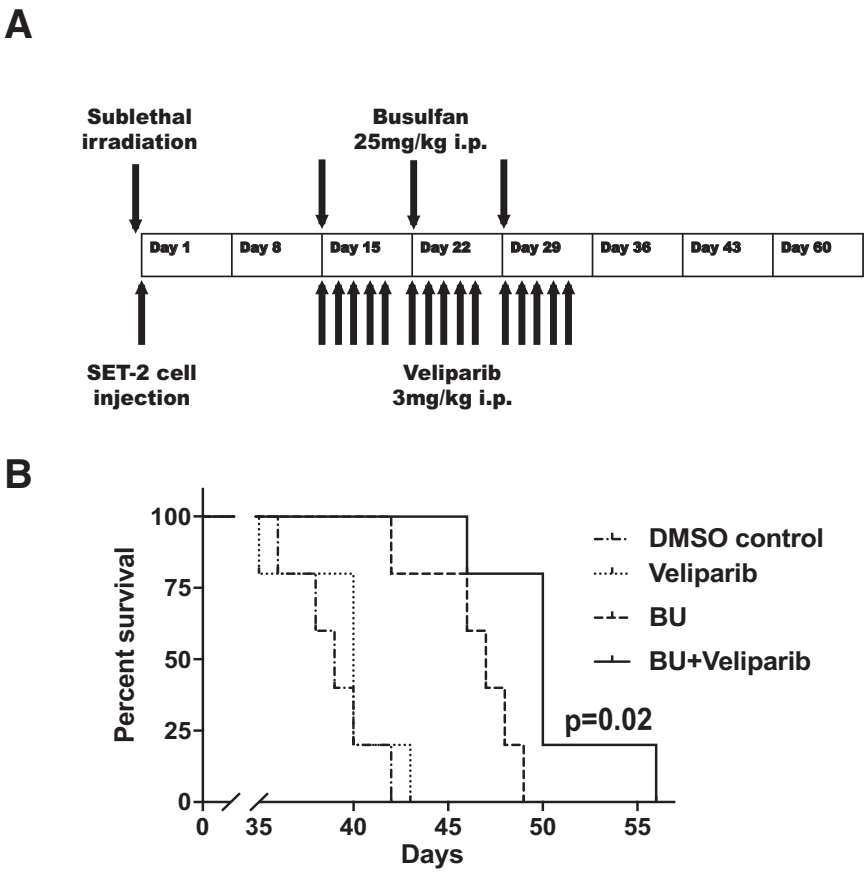

Figure 2. (A) Treatment schema and survival of the NOD/SCID/gamma null (NSG) xenograft model of MPN (using SET2 cells) used to investigate the in vivo treatment effect of veliparib and busulfan. To establish disease, 5×10^6 SET2 cells were injected via the tail vein into NSG mice after sublethal irradiation. The i.p. drug treatment was started 14 days after SET-2 injection and continued for a total of 3 weeks or less in mice that died earlier. Mice (n = 5 per group) were treated with vehicle, veliparib alone (3 mg/kg) for 5 days a week, once-weekly busulfan alone (25 mg/kg), or a combination of the 2 drugs. (B) Median survival of mice treated with veliparib and busulfan combinations. Vehicle- and veliparib-treated mice had similar median survival, 39 and 40 days, respectively. There were significant differences among the curves ($P < .0001$); specifically, combination treatment increased median survival from 47 days (busulfan alone) to 50 days ($P = .02$).

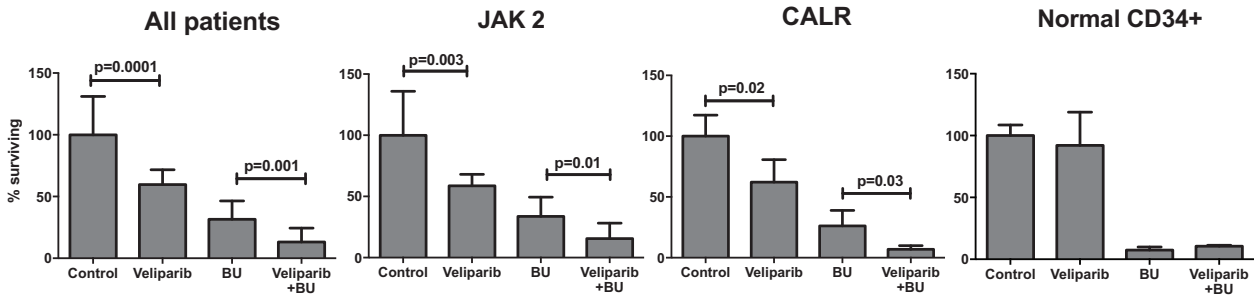

Figure 3. Colony formation as a percentage of control in CD34$^+$ cells from patients with MF (n = 7) and normal patients (n = 3) after treatment with veliparib, busulfan, or a combination of the 2 drugs. Veliparib significantly reduced CFU-C from MF CD34$^+$ cells, but not from normal CD34$^+$ cells. In addition, the combination of busulfan and veliparib led to a significant decrease in CFU-C compared with the use of either drug alone. Findings were noted in both JAK2-mutated MF (n = 5) and CALR-mutated MF (n = 2).

DISCUSSION

Here we report that treatment with the PARP inhibitor veliparib and busulfan results in synergistic cytotoxicity in MPN cell lines and primary MPN cells. The enzyme PARP-1 has a primary role in base excision repair (BER) but is also involved in HR [8]. Specifically, PARP-1 is activated by and binds to stalled replication forks, where it recruits HR repair enzymes to restart DNA replication [9,10]. PARP inhibition prevents activity but also leads to trapping of PARP-1 at the site of DNA damage, further inhibiting recruitment of HR repair enzymes to stalled replication forks [11]. Since *JAK2*^{V617F} mutated cells have been demonstrated to show baseline DNA damage [12,13], spontaneous HR [6,7], and replicative fork stalling [5], DNA repair inhibition represents a potential novel therapy. Furthermore, because busulfan is known to slow and stall replication forks and is integral in pretransplantation conditioning, combination with PARP-1 inhibition in MPNs has a strong rationale. Although previous data on the genomic instability of MPNs is largely restricted to *JAK2*-mutated cells, here we show that *CALR*-mutated cells show similar sensitivity to veliparib, suggesting that PARP inhibition may be a viable treatment for MPNs regardless of mutational status.

To date, PARP inhibitors have been approved and used successfully as single agents in the treatment of ovarian cancer [14,15] and *BRCA*-associated breast cancers [15]. In addition, several trials in breast, ovarian, and cervical cancers have combined PARP inhibitors with cytotoxic chemotherapy requiring PARP-1 activity for DNA repair, including alkylating agents, topoisomerase I inhibitors, and platinum-based drugs with some modest signs of increased efficacy [16-19]. Two separate Phase I trials have evaluated the safety of the addition of veliparib to chemotherapy in aggressive myeloid neoplasms [20,21]. Of note, patients with aggressive MPNs had a relatively high rate of complete remission when treated with a triplet combination of veliparib, carboplatin, and topotecan [20]. Our data build on the current preclinical and clinical data and provide the rationale for testing novel pretransplantation conditioning regimens with combinations of PARP-1 inhibition and reduced doses of alkylators, such as busulfan and melphalan. Furthermore, our data have wider implications for the treatment of high-risk MPNs and MPN-AML. Combination therapies including DNA repair inhibition could be further investigated for the treatment of these patients.

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Authorship statement: P.R.P. and D.R. contributed to the study design and analyzed all data; P.R.P., V.S., D.M., A.O., E.B., and N.M. performed the experiments; P.R.P., G.B., N.M., and D. R. contributed to the writing of the manuscript; and all authors assisted in the critical review of the manuscript and approved the final version of the manuscript for submission.

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