A Cyclin-Dependent Kinase Inhibitor, Dinaciclib, Impairs Homologous Recombination and Sensitizes Multiple Myeloma Cells to PARP Inhibition

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Abstract

PARP1/2 are required for single-strand break repair, and their inhibition causes DNA replication fork collapse and double-strand break (DSB) formation. These DSBs are primarily repaired via homologous recombination (HR), a high-fidelity repair pathway. Should HR be deficient, DSBs may be repaired via error-prone nonhomologous end-joining mechanisms, or may persist, ultimately resulting in cell death. The combined disruption of PARP and HR activities thus produces synthetic lethality. Multiple myeloma cells are characterized by chromosomal instability and pervasive DNA damage, implicating aberrant DNA repair. Cyclin-dependent kinases (CDK), upstream modulators of HR, are dysregulated in multiple myeloma. Here, we show that a CDK inhibitor, dinaciclib, impairs HR repair and sensitizes multiple myeloma cells to the PARP1/2 inhibitor ABT-888. Dinaciclib abolishes ABT-888-induced BRCA1 and RAD51 foci and potentiates DNA damage, indicated by increased γH2AX foci. Dinaciclib treatment reduces expression of HR repair genes, including Rad51, and blocks BRCA1 phosphorylation, a modification required for HR repair, thus inhibiting HR repair of chromosome DSBs. Cotreatment with dinaciclib and ABT-888 in vitro resulted in synthetic lethality of multiple myeloma cells, but not normal CD19+ B cells, and slowed growth of multiple myeloma xenografts in SCID mice almost two-fold. These findings support combining dinaciclib with PARP inhibitors for multiple myeloma therapy.

Introduction

Multiple myeloma is a hematologic malignancy characterized by clonal plasma cell proliferation in the bone marrow (1), and widespread chromosomal instability in those cells (2). Although the mechanisms underlying genomic instability in multiple myeloma are not clearly defined, deranged DNA repair mechanisms have been implicated (3–5). Cells employ several DNA repair mechanisms to withstand genotoxic stress. The most lethal DNA lesions, double-strand breaks (DSB), are repaired by one of two major pathways, nonhomologous end-joining (NHEJ) and homologous recombination (HR). The mechanism of NHEJ is simpler (direct ligation of DSB ends), but the process is more error prone in the absence of sequence homology to guide repair (6). HR repair instead uses a sister chromatid or more rarely, a non-sister homolog, as template for high-fidelity repair, and thus occurs mainly in the S and G2 cell-cycle phases when a sister chromatid is available (7).

During HR repair, DSBs can be detected by the MRE11–RAD50–NBS1 (MRN) complex, which recruits and activates ATM (8). Activated ATM phosphorylates multiple proteins involved in initiating repair and checkpoint arrest, including H2AX, MDC1, CHK2, and BRCA1 (8–10). Cyclin-dependent kinase (CDK)-mediated phosphorylation activates CIP, enabling it to interact with BRCA1 to promote resection of DSB termini, thus generating 3′-single-strand DNA (ssDNA) tails (11, 12). With the aid of BRCA2, RAD51 binds to ssDNA to form RAD51 nucleofilaments, which orchestrate a homology search on a template (e.g., a sister chromatid), strand invasion, and repair of the DSB (13).

SSBs in DNA are repaired via the base or nucleotide excision repair pathway and require PARP1, and PARP2 to some extent (14). Inhibition of PARP1/2 therefore leads to accumulation of SSBs that cause collapse of DNA replication forks and formation of lethal DSBs (15). These replication-associated DSBs are primarily repaired via HR, the high-fidelity repair pathway for DSBs (16). In the absence of HR, the DSBs persist, unless repaired by error-prone NHEJ mechanisms, ultimately resulting in cell death (14, 17, 18). Synthetic lethality thus exists between PARP1 and HR functions, which has been exploited to selectively kill cells that harbor HR deficiencies due to mutations in their BRCA genes (15, 18). Expression of PARP1 and the frequency of HR are both elevated in multiple myeloma cells (3, 19), whereas defective DNA repair mechanisms, including NHEJ (4, 20) and mismatch repair (MMR; ref. 5), have been reported. Unlike normal bone marrow plasma cells and normal B cells, multiple myeloma cell lines and patient plasma cells exhibit pervasive DNA damage and rearrangements (21). Thus, multiple myeloma cells may be highly dependent on PARP1 and HR functions to survive.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-15-0660
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Molecular Cancer Therapeutics
oncogene-induced replication stress and the DNA damage that ensues. We recently reported synergy between DNA-damaging agents and HR inhibitors in their toxicity to myeloma cells (see Discussion), which may be mechanistically related to contextual synthetic lethality shown for simultaneous inhibition of HR and proteasomal degradation (19).

Recent studies have revealed critical roles for CDKs in regulating HR; however, CDK dysregulation is a hallmark of multiple myeloma cells and CDKs have been designated as therapeutic targets (22–24). CDK1 and CDK2 phosphorylate BRCA1 on serine 1497 (S1497; refs. 25, 26) and in addition, CDK1 phosphorylates BRCA1 on S1189/1191 (26). These posttranslational modifications are important for formation of BRCA1 and RAD51 foci in response to DNA damage and HR repair (26, 27). Also, CDKs and its activator, CDK5R/p35, are overexpressed in multiple myeloma cells relative to normal somatic tissue (28, 29). This CDK can phosphorylate and activate ATM, which is critical for activation of the intra-S and G2–M checkpoints following DNA damage (28, 30). Consequently, cancer cells depleted of either CDK1 or CDK5 becomes hypersensitive to PARP inhibitors (28, 31), whereas upregulation of CDK5 can mediate chemoresistance (32). Because of the critical roles CDKs play in DNA repair by HR, CDK inhibitors such as flavopiridol, AG024322, and AZD5438 have been shown to impair HR function and sensitize cancer cells to DNA-damaging agents, including PARP inhibitors (33–35).

Dinaciclib is a potent small-molecule inhibitor of CDKs 1, 2, 5, and 9 (36). It has shown promising results in early-phase clinical trials for multiple myeloma (24). In this study, we postulated that dinaciclib could disrupt HR repair in multiple myeloma cells, leading to a contextual synthetic lethality if combined with PARP1/2 inhibition. We here provide evidence that dinaciclib does impair HR repair efficiency in multiple myeloma cells and sensitizes them to PARP inhibition both in vitro and in vivo.

Materials and Methods

Cell culture and treatments

The human multiple myeloma cell lines NCI-H929, RPMI-8226, and MM.1S were purchased from ATCC and authenticated by us, were main- tained in the same medium but supplemented with 2 μg/mL puromycin (Sigma Aldrich). Normal human peripheral blood CD19+ B cells (ZenBio) were cultured in ZenBio Lymphocyte medium.

ABT-888 (Santa Cruz Biotechnology, Inc.), doxorubicin-HCl (Sigma Aldrich), and dinaciclib (SCH227965; ChemieTek) were dissolved in DMSO. The stock concentrations were diluted in cell culture medium prior to cell treatment. For in vitro studies, dinaciclib was dissolved in 1% Captisol (Ligand Pharmaceuticals, Inc.).

Flow cytometry analysis of cell-cycle distributions, bromodeoxyuridine incorporation, and histone H3 (S10) phosphorylation

After the indicated treatments, cell-cycle distribution was analyzed, and phosphorylated histone H3 (pHH3; phosphorylated at residue S10) was visualized by immunostaining as previously described (37).

For propidium iodide (PI) and bromodeoxyuridine (BrdUrd) dual staining, cells were incubated with 10 μmol/L BrdUrd (Sigma) at 37°C for 1 hour, fixed in 70% ethanol, denatured in 2 mol/L HCl, and neutralized in 0.1 mol/L sodium borate. Cells were then stained with FITC-labeled antibody to BrdUrd (BD Pharmigen), resuspended in 500 μL PBS containing 25 μg/mL PI and 1.25 mg/mL RNase A, and incubated at 37°C for 30 minutes in the dark. Nuclear staining was then quantified by flow cytometry (FACScan), using FlowJo 4.4.4 software for DNA content analysis.

RT-qPCR

Total RNA was extracted from cells after the indicated treatments using RNAeasy Mini Kit (Qiagen). Total RNA (1 μg) was used to reverse transcribe cDNA using SuperScript First Strand cDNA Synthesis Kit (Invitrogen). The cDNA was amplified by RT-qPCR using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The amplified PCR products were detected using SYBR Green Master Mix (Roche). The primers used for amplification were:

**Rad51**: 5′-CAATGCAGATGCAGCTTGA-3′ (F) and 5′-CCTTG-GCTTCACATTCCCT-3′ (R).

**Rad51b**: 5′-TTTCCCCACTGAGAGCTG-3′ (F) and 5′-CCTTGC-CCAAAGCAGAAAG-3′ (R).

**Rad51c**: 5′-AGAGCTTCCGCTTGAATG-3′ (F) and 5′-GGAGTT-CCTCACGCTTG-3′ (R).

**Rad51d**: 5′-AGTTGGACCGCTGTTTGC-3′ (F) and 5′-CCAGTG-CCGCGCTTC-3′ (R).

**Xrcc2**: 5′-TAGTTGCTTTCAATAGGGCTGA-3′ (F) and 5′-TGGGA-AGTATACATCGTGTTG-3′ (R).

**Xrcc3**: 5′-AAGAAAGTCCCGTACTGCTG-3′ (F) and 5′-CTGTCG-CCTGTTCACTG-3′ (R).

**Brcal**: 5′-TAGGGCTGGAAGCAGAAGT-3′ (F), 5′-ATTTCCCT-CCCGAATGTTCC-3′ (R).

**Gapdh**: 5′-GTCCACTTGGCGCTTCACCA-3′ (F), 5′-GGGCCAGT-GATGGCATGGAC-3′ (R).

**Western blots**

After the indicated treatments, whole-cell lysates were prepared in cell lysis buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific). The supernatants were collected after centrifugation at 14,000 rpm at 4°C for 10 minutes. Protein concentrations were determined using the BCA Assay Kit (Thermo Scientific). Equal protein loads, from all samples in an experiment, were heated for 5 minutes at 95°C in Laemmli buffer (Bio-Rad), resolved by SDS-PAGE, transferred to a polyvinyl difluoride membrane (Millipore), and probed with antibodies recognizing RAD51 (mouse monoclonal IgG, Millipore) and p-BRCA1 (S1497, rabbit polyclonal IgG, Santa Cruz Biotechnology). Membranes were
Synthetic Lethality between CDK and PARP Inhibition

HR repair assay
The effect of dinaciclib on HR repair was assessed as previously described (37). Cells were incubated with primary antibodies against γH2AX (ser139; 1:1,000 mouse monoclonal IgG, clone JBW301, Millipore) and either RAD51 (goat polyclonal IgG; Santa Cruz Biotechnology) or BRCA1 (1:1,000 rabbit polyclonal IgG, Santa Cruz Biotechnology) at 4 °C overnight. After three 5-minute washes with PBS, cells were incubated for 1 hour at 22 °C in the dark, with appropriate secondary antibodies at 1:1,000 dilutions [bovine anti-goat IgG Alexa Fluor 488 for RAD51 or donkey anti-rabbit IgG FITC and goat anti-mouse IgG Alexa Fluor 594 for γH2AX (Jackson Immuno-Research)]. Cells were washed three times in PBS and mounted under coverslips with Prolong Antifade reagent containing DAPI. Images were acquired with an LSM 510 Zeiss confocal laser-scanning microscope with a 63× oil objective. For quantitative analysis, ≥100 cells from each group were chosen at random and nuclei were (i) counted manually to determine the percent positive for BRCA1 or RAD51 or β-actin (Santa Cruz Biotechnology) and detected as above. RAD51 and pBRCA1 protein band densities were quantified using Quantity One software (Bio-Rad) and the values expressed relative to actin and total BRCA1, respectively (controls for loading and transfer). The protein levels in treated samples were standardized against controls exposed only to DMSO. At least two independent experiments were performed for each comparison.

Cell proliferation and colony formation assays
Cell proliferation/viability and colony formation assays were performed as previously described (37). The percent viability of cells was calculated relative to vehicle treatment (set as 100% viability for dose–response curves).

Myeloma xenograft mouse model
The animal study was approved by the Animal Use and Care Committee of the University of Arkansas for Medical Sciences (Little Rock, AR). The xenograft model used here has been described previously (38). Briefly, CB-17/SCID male mice (Harlan Laboratories Ltd.) were subcutaneously inoculated with 5.0 × 10⁶ MM.1S cells in 5% Matrigel (Corning) in serum-free RPMI1640 cell culture medium. Approximately 2 weeks after cell inoculation, when tumors became measurable (100–150 mm³), mice were assessed for initial tumor size, randomized into four groups (each N = 10), and treated as follows:

1. DMSO [6%, v/v, intraperitoneally (i.p.) twice per week]
2. Dinaciclib (35 mg/kg i.p. twice per week; ref. 36)
3. ABT-888 (50 mg/kg by oral gavage, twice daily, 5 days per week; ref. 19)
4. The combination of (2) and (3).

The long-axis (a) and short-axis (b) diameters of tumors were measured by caliper every third day to estimate tumor volume using the formula: V = 0.5a × b² (19). For survival analysis, survival time was defined as time required for tumor volume to reach a set endpoint, that is, tumor growth exceeding 2 cm across the long axis, or causing major distress to the mice. Differences in tumor volume among treatment groups were tested for significance by one-way ANOVA with Tukey post hoc tests. Survival was determined from Kaplan–Meier curves, as the time from the first day of treatment until mice were sacrificed (based on the above criteria). Statistical significance of survival differences was determined by a log-rank (Mantel–Cox) test.

Statistical analysis
GraphPad Prism software (Prism ver. 6) and Microsoft Excel were used for statistical analysis. Statistical significances of differences between groups were calculated by the Fisher test, requiring P < 0.05 for nominal significance.

Results
Cotreatment with the CDK inhibitor dinaciclib and the PARP1/2 inhibitor ABT-888 causes synthetic lethality of human myeloma cell lines, but not of normal peripheral B cells
Treatment of multiple myeloma cells with the PARP1/2 inhibitor ABT-888 has been shown to induce DNA DSBs that are effectively repaired via HR, and therefore do not affect cell viability (19). Because we hypothesized that the CDK inhibitor dinaciclib would impair HR repair and hence sensitize multiple myeloma cells to PARP inhibition, we first assessed cell viability under growth conditions, and clonogenic survival at low density, of multiple myeloma cells treated with ABT-888 or dinaciclib as single agents versus their combination. As shown in Fig. 1A–C, E, and F, treatment with ABT-888 (2.5–20 nmol/L) alone had no significant effect (less than 10%; each P > 0.05) on cell viability in either assay, for any of the multiple myeloma cell lines tested. Dinaciclib was also tested across a dose range (5–40 nmol/L) but data are shown here only for 20 nmol/L (a dose slightly above the IC₅₀ which produced maximum sensitization to the PARP inhibitor). Dinaciclib caused significant inhibitions of both viability and colony formation, which were similar (47%–55% reduction; each P ≤ 0.01) for all of the multiple myeloma cell lines tested. The combination of dinaciclib with ABT-888, however, produced synergistic effects on cell survival (synthetic lethality), similarly dependent on ABT-888 dose for every multiple myeloma cell line (Fig. 1A–C). A statistical criterion for synergy, that the viable fraction for dual treatment must be significantly lower (at P < 0.05) than the product of viable fractions after individual treatments, was met for 9 of the 12 non-zero ABT-888 treatments (Fig. 1A–C) in mass culture conditions, and for both combined treatments under clonal growth conditions (Fig. 1E and F). We observed similarly pronounced synergistic effects when dinaciclib was combined with low doses of doxorubicin (20–160 nmol/L), washed in TBST, incubated for 1 hour at 22 °C with horseradish peroxidase–conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology), and washed again. Protein signals were detected by chemiluminescence using an ECL Detection Reagent (Bio-Rad). After removing antibodies in stripping buffer [2% w/v SDS; 62.5 mmol/L Tris-HCl pH 6.8; and 0.7% (w/w) β-mercaptoethanol] for 15 minutes at 50 °C, the membrane was probed with primary antibodies to BRCA1 and β-actin (Santa Cruz Biotechnology) and detected as above. RAD51 and pBRCA1 protein band densities were quantified using Quantity One software (Bio-Rad) and the values expressed relative to actin and total BRCA1, respectively (controls for loading and transfer). The protein levels in treated samples were standardized against controls exposed only to DMSO. At least two independent experiments were performed for each comparison.
Dinaciclib impairs recruitment of BRCA1 and RAD51 proteins to DSB sites and reduces HR repair of chromosome DSBs in multiple myeloma cells

As noted above, ABT-888 alone induces DSBs in multiple myeloma cell DNA, but these are efficiently repaired (19). Because CDKs 1, 2, and 5 (inhibited by dinaciclib) are known to play key roles in DSB repair, we asked whether dinaciclib might owe its synthetic lethality in combination with ABT-888, to blockage of HR repair allowing persistence of ABT-888–induced DSBs. To this end, we used immunofluorescence detection and confocal microscopy to assess the levels of nuclear BRCA1 and RAD51 foci, which are indicators of HR repair, and of γH2AX foci (a marker of DSBs), after treatment of multiple myeloma cells with DMSO control, dinaciclib, ABT-888, or dinaciclib plus ABT-888. As shown in Fig. 2B, dinaciclib prevented repair of ABT-888–induced DNA damage, thus significantly reducing the fraction of cells with ≥5 nuclear BRCA1 foci from 47% to 6% (P < 0.0001) and ≥5 nuclear RAD51 foci from 46% to 4% (P < 0.0001), while causing a marked increase in the fraction of cells with ≥5 nuclear γH2AX foci from 19% to 52% (P < 0.0001). The integral of fluorescence signal per nucleus paralleled quite well the fraction of cells exceeding an arbitrary threshold (Fig. 2). It is also noteworthy that dinaciclib treatment alone induced significant DNA damage, increasing the basal levels of cells with ≥5 nuclear γH2AX foci from 20% to 35% (P < 0.05). This could be due to inhibition of CDK2, which is known to activate components of DSB repair signaling, including formation of γH2AX foci (39–41).

We next assessed the direct impact of dinaciclib on the repair of chromosome DSBs via HR. The MM.1S cell line has robust HR repair capability (3, 37) and so we used MM.1S cells bearing a chromosomally integrated DR-GFP reporter construct to measure HR repair as described previously (19, 37). Typical results, shown in Fig. 3, indicate 19% GFP+ cells (cells with HR repair events following induction of a target-specific DSB) in control cells, but only 3% in dinaciclib-treated reporter cells, a 84% decrease in HR repair after dinaciclib treatment (P < 0.0001). Taken together, these findings demonstrate that dinaciclib impairs the recruitment of BRCA1 and RAD51 to DNA DSB sites, and subsequently inhibits HR repair of chromosomal DSBs.

Dinaciclib lowers the mRNA levels of Rad51, its paralog, and Brca1, and reduces the protein levels of RAD51 and damage-induced phosphorylated BRCA1

The RAD51 complex plays a central role in HR repair; RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) and BRCA1 promote RAD51 function during DSB repair (13). CDK inhibitors can induce p53-mediated downregulation of RAD51 at both the mRNA and protein levels, resulting in HR impairment and hypersensitivity of cells to DNA-damaging agents (42, 43). CDK-mediated phosphorylation of BRCA1 on S1497 is required.
Dinaciclib induces S-phase accumulation and reduces G2-M phase arrest

Cell-cycle regulation is a major role of CDKs (44), which could in turn influence the expression of DNA repair genes and the choice of DNA repair pathways. Because we have demonstrated that dinaciclib disrupts HR repair, which occurs predominantly during the S- and G2 cell-cycle phases, we wanted to rule out possible preclusion of HR repair due to G1 arrest. We therefore used PI staining to assess the cell-cycle distribution after exposure to dinaciclib, and/or doxorubicin, which causes multiple myeloma cell-cycle arrest in S and G2 (37). Treatment with doxorubicin for 24 hours caused cells to arrest in the S- and G2 phases, as expected (Fig. 5A), with very few cells in G1. Treatment with dinaciclib (SCH) alone caused moderate increases of cells in S-phase (from 20% to 30%), and G1 (from 50% to 58%), but a much more pronounced decrease (from 31% to 12%) in G2-M phase cells. This effect was observed for all of the multiple myeloma cell lines tested (Supplementary Fig. S2A and S2B). As the total decrease of cells in the sum of S and G2 phases together was just 8% to 9%, this would not account for the >5-fold impairment of HR by dinaciclib. The doxorubicin-induced G2-M accumulation was completely reversed by dinaciclib, which restored SCH + doxorubicin-treated cells to nearly the same distribution as SCH treatment alone, for example, G2-M shifted from 63% to 16% (Fig. 5). We also assessed the cell-cycle effects of ABT-888 with or without dinaciclib. Compared with control DMSO treatment, ABT-888 caused an increase in G2–M phase (from 31% to 45%) and a corresponding decrease of cells in G1 phase (from 50% to 33.6%). The G2 phase accumulation was similarly reversed when dinaciclib was added to ABT-888 (data not shown), just as when it was combined with doxorubicin (Fig. 5).

Because dinaciclib caused cells to accumulate in S-phase, with a marked decrease in G2–M cells, but abrogated doxorubicin-induced G2–M phase accumulation, we assessed the degree of S-phase arrest by BrdUrd incorporation, and stained cells for phospho-histone H3 (pHH3, phosphorylated at Ser10) to determine whether the G2 checkpoint was abrogated. In fact, very few cells treated with dinaciclib and/or doxorubicin underwent DNA damage (26, 27). We therefore assessed transcript levels of RAD51 and γH2AX foci in cells exposed to drugs indicated at left. B, mean percent of cells with >5 RAD51 or γH2AX foci, ±SEM, after the exposures indicated; data were combined from three experiments. C, integrated signal intensity per nucleus, after subtraction of peripheral background. * * * * , and ****, P < 0.05, P < 0.01, P < 0.001, and P < 0.0001, respectively, for two-tailed t tests on the effect of drug treatment relative to DMSO (vehicle).

A

Figure 2. Dinaciclib impairs recruitment of BRCA1 and RAD51 proteins to DSB sites. MM.1S cells, exposed for 24 hours to DMSO, dinaciclib (SCH; 20 nmol/L), ABT-888 (ABT; 20 μmol/L), or SCH + ABT, were examined by immunofluorescence to identify foci, and DAPI staining to define nuclei. A, representative images of RAD51 and γH2AX foci in cells exposed to drugs indicated at left. B, mean percent of cells with >5 RAD51 or γH2AX foci, ±SEM, after the exposures indicated; data were combined from three experiments. C, integrated signal intensity per nucleus, after subtraction of peripheral background. * * * * , and ****, P < 0.05, P < 0.01, P < 0.001, and P < 0.0001, respectively, for two-tailed t tests on the effect of drug treatment relative to DMSO (vehicle).
synthesis (i.e., the BrdUrd-positive fraction of cells in S-phase was <2%, Fig. 5D and F). Thus, the S-phase checkpoint was apparently not inactivated by dinaciclib. Phospho-histone H3 is a marker of mitotic cells, and often used to distinguish cells in G2 and M phases and thus to determine G2 checkpoint abrogation. Cells treated with dinaciclib and/or doxorubicin showed very little pHH3 staining (<0.015%), indicating that cells in the G2–M phase were almost entirely arrested in G2. Thus, there was little G2 checkpoint abrogation.

We thus conclude from our cell-cycle data that the impairment of HR by dinaciclib could not be explained by G1 arrest. On the contrary, accumulation of cells in S-phase could enhance drug cytotoxicity as cells are most vulnerable to killing by cytotoxic drugs such as anthracyclines (e.g., doxorubicin) during the S-phase (45).

Cotreatment with dinaciclib and ABT-888 delays tumor growth and significantly prolongs survival of SCID mice bearing multiple myeloma xenografts

We next evaluated the effects of ABT-888 (50 mg/kg by oral gavage twice daily, 5 days per week) and/or dinaciclib (35 mg/kg i.p., twice per week) on the growth of MM.1S cells as subcutaneous tumors, after subcutaneous inoculation in male SCID mice. On the third week of treatment, tumor volume had increased 20-fold in mice exposed only to vehicle (6% DMSO), and 19-fold for ABT-888, but 9-fold with dinaciclib (P < 0.01 compared with untreated controls or ABT-888 treatment), and just 1.7-fold for cotreatment with dinaciclib and ABT-888 (P < 0.012, compared with either single agent; Fig. 6). The in vivo effects were thus in line with the in vitro data. Log-rank tests for Kaplan–Meier survival curves also showed a significant increase in premorbid survival (P ≤ 0.004; Fig. 6) for the dinaciclib plus ABT-888 cotreated group relative to either single agent. Although we observed mild diarrhea and slight weight loss in mice treated with dinaciclib, they were overall very healthy and active and did not show signs of discomfort. To further assess whether the downregulation of DNA repair genes after dinaciclib treatment also occurs in vivo, when tumors had reached the predetermined size limit (2,000 mm3), we repeated the drug treatments for 24 hours, then excised the tumors, and preserved them in RNAlater for RT-qPCR analyses on total RNA. We observed significant downregulation of mRNA levels for Rad51, Xcc3, and Brcat in groups treated with dinaciclib.
alone or combined with ABT-888 (Supplementary Fig. S3). The reductions in transcript levels for Rad51b, Rad51c, Rad51d, and Xrc2 were not statistically significant after dinaciclib, and ABT-888 treatment had no significant impact on the expression of any of the genes tested.

In summary, our in vivo data on tumor growth and transcript levels were entirely consistent with the in vitro data (although somewhat more variable), lending further support to our hypothesis that contextual synthetic lethality to multiple myeloma cells can be achieved with the combination of dinaciclib and ABT-888.

**Discussion**

Unlike normal bone marrow plasma cells and normal B cells, multiple myeloma cell lines and patient plasma cells display marked chromosomal instability (2) and pervasive DNA damage (21). These implicate aberrant DNA repair pathways in the disease. We have previously reported that HR frequency is elevated in multiple myeloma and contributes to genomic instability and disease progression (3), while other studies have shown that PARP1 is overexpressed and correlates with shortened survival (19). Elevated HR and PARP1 activities may compensate for certain defects in components of the DNA damage response machinery common to myeloma cells, such as NHEJ (4) and MMR (5, 46); thus myeloma cells may be especially dependent on PARP1 and HR for survival (19, 46). Recent studies have identified critical roles for CDKs in HR repair, and specific depletion of CDK1 or CDK5 has been shown to cause hypersensitivity of tumor cells to PARP inhibitors (26–28). We have demonstrated in this study that the CDK inhibitor dinaciclib disrupts HR function and causes a contextual synthetic lethality of multiple myeloma cells when combined with the PARP1/2 inhibitor ABT-888, while sparing normal peripheral blood CD19+ B cells.

We demonstrated that the survival of multiple myeloma cell lines is not significantly impaired by treatment with the PARP inhibitor ABT-888 alone. This is not surprising, considering the elevated state of HR in multiple myeloma cells (3). Moreover, previous studies demonstrated that ABT-888 does not affect...
Dinaciclib induces S-phase accumulation and reduces G2-M phase accumulation. MM.1S cells were treated as indicated for 24 hours, and cell-cycle distribution was determined by propidium iodide (PI) staining (A and B). C and D, BrdUrd incorporation was assessed to determine the percent of cells with S-phase DNA content (as determined by PI staining) that are BrdUrd-positive or negative. E and F, after the indicated treatments, cells were costained for DNA content with PI, and for phospho-histone H3 (S10) with Alexa Fluor 488–conjugated antibody to phospho-histone H3 (S10) with Alexa Fluor 488–conjugated antibody to phospho-histone H3 (S10). E, representative flow cytometry scatter plots. F, averaged results are shown for at least P < 0.001. DOX, doxorubicin.

We posited that HR deficiency and the consequent increase in DNA damage are the basis for dinaciclib-induced sensitivity of multiple myeloma cells to PARP inhibition. In keeping with this, we demonstrated that ABT-888 treatment induced BRCA1 and RAD51 foci, which are indicative of proficient HR repair, thereby resulting in very little DNA damage or few γH2AX foci. On the contrary, upon combining ABT-888 with dinaciclib, the reverse was true: fewer BRCA1 and RAD51 foci and increased γH2AX foci were observed. The inhibition of HR repair was further supported by our GFP-based HR assays, which showed that dinaciclib directly blocks HR repair of chromosomal DSBs. To pursue this, we examined the basis for reduced BRCA1 and RAD51 foci induced by dinaciclib, and found that dinaciclib impairs phosphorylation of BRCA1 on ser1497, the expected CDK phosphorylation site (25, 26). Dinaciclib also decreased both Rad51 mRNA and protein levels of RAD51, a core protein essential for HR repair whose depletion sensitizes tumor cells to PARP inhibition (49). In addition, the transcript levels of Rad51 paralogs as well as Brcal were reduced by dinaciclib. These genes encode proteins that promote RAD51-mediated HR repair. The decrease in RAD51 transcripts and protein was in line with previous findings that CDK inhibitors mediate p53-dependent downregulation of RAD51 at both the transcript and protein levels, and sensitize cancer cells to DNA-damaging agents (42, 43). We therefore conclude that these effects of dinaciclib led to the impaired HR function and the subsequent sensitivity of multiple myeloma cells to PARP inhibition as well as doxorubicin. Moreover, we have previously reported that disruption of RAD51 expression, via siRNA, peptide nucleic acid, or a small-molecule inhibitor, impairs HR function and sensitizes multiple myeloma cells (37, 50). We also note that CDK1/2-mediated phosphorylation of CtIP is required for HR repair, and inhibition of these CDKs via triapine has been demonstrated to compromise HR repair and sensitize DNA damage and defects in DNA repair pathways, leading to especially high dependence on HR and PARP activities. In contrast, normal cells have intact DNA repair pathways and accumulate little or no unrepaired DNA damage. Thus, inhibiting PARP and HR would be expected to show preferential toxicity for multiple myeloma cells. This provides a good rationale for combining dinaciclib and ABT-888 in multiple myeloma treatment, as this would widen the therapeutic window. With regard to the differential sensitivity to dinaciclib, of multiple myeloma cell lines relative to normal B cells, deregulation of the cell cycle and the high proliferation of multiple myeloma cell lines could increase their sensitivity to CDK inhibitors compared with normal cells. In addition to PARP inhibition, we found that dinaciclib produced synergistic effects in combination with doxorubicin, a topoisomerase II inhibitor. Previous studies have shown that dinaciclib enhances the antitumor effects of gemicitabine, an agent known to cause replication fork collapse and DSB formation, which requires HR repair (47). Also, Raje and colleagues reported that seliciclib (a CDK inhibitor with targets similar to dinaciclib), produced synergistic toxicity for multiple myeloma cells when combined with doxorubicin (48). These studies are consistent with our observations that dinaciclib potentiates the anti-multiple myeloma effects of ABT-888, which causes replication fork collapse and subsequent DSB formation, and doxorubicin, which induces DSBs.

Multiple myeloma cell survival because their HR efficiently repairs the induced DSBs (19). Although we found that dinaciclib alone significantly decreased multiple myeloma cell viability, a synthetic lethal effect ensues when it is combined with the PARP inhibitor ABT-888, leading to a significantly greater loss of viability relative to these agents given singly, and even relative to the product of their individual effects. This synthetic lethal effect was recapitulated in multiple myeloma xenografts in SCID mice, where tumor growth was significantly delayed and survival prolonged for the combination compared with individual agents. Intriguingly, a synthetic lethal effect was not observed in normal B cells. As mentioned above, multiple myeloma cells have ongoing
to reduce HR repair in addition to the pathways we investigated. Finally, we have demonstrated that the HR repair–inhibitory effects of dinaciclib are not cell-cycle related, as dinaciclib did not induce cell-cycle phase arrest in G1, which, if it occurred, could preclude HR repair. It remains to be determined which specific CDK(s) inhibited by dinaciclib account for its HR-inhibitory effects in multiple myeloma cells.

To summarize, we have used a CDK inhibitor, dinaciclib, to disrupt BRCA1 and RAD51 functions, which impaired the ability of multiple myeloma cells to repair chromosome DSBs via HR. This approach selectively sensitizes multiple myeloma cells to the PARP1/2 inhibitor ABT-888, in a manner that (based on similar synthetic lethality for other drug combinations) appears to be mediated by dinaciclib’s disruption of HR repair. As CDKs, PARP1, and HR are deregulated in multiple myeloma and have been designated as therapeutic targets, our findings provide a strong rationale for combining dinaciclib with PARP inhibitors in targeting multiple myeloma cells, and a basis for further investigations of their safety and efficacy in multiple myeloma patients.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.A. Alagpulinsa

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.A. Alagpulinsa, S. Ayyadevara, S. Yaccoby, R.J. Shmookler Reis

Writing, review, and/or revision of the manuscript: D.A. Alagpulinsa, S. Ayyadevara, R.J. Shmookler Reis

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Yaccoby

Study supervision: S. Ayyadevara, S. Yaccoby, R.J. Shmookler Reis

**Grant Support**

This study was supported by a VA Merit grant [Principal Investigator (PI): R.J. Shmookler Reis], and NIH grants P01 CA055819-20 [PI: B. Barlogie (PL: S. Yaccoby)] and P30 AG028718 [PI: J. Wei (CL: R.J. Shmookler Reis)].

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Received August 10, 2015; revised October 21, 2015; accepted November 9, 2015; published OnlineFirst December 30, 2015.

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**References**


A Cyclin-Dependent Kinase Inhibitor, Dinaciclib, Impairs Homologous Recombination and Sensitizes Multiple Myeloma Cells to PARP Inhibition

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