Targeting the Fanconi Anemia/BRCA Pathway Circumvents Drug Resistance in Multiple Myeloma

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Abstract

The Fanconi anemia/BRCA (FA/BRCA) DNA damage repair pathway plays a pivotal role in the cellular response to replicative stress induced by DNA alkylating agents and greatly influences drug response in cancer treatment. We recently reported that FA/BRCA genes are overexpressed and causative for drug resistance in human melphalan-resistant multiple myeloma cell lines. However, the transcriptional regulation of the FA/BRCA pathway is not understood. In this report, we describe for the first time a novel function of the NF-κB pathway in response to chronic melphalan administration—approved agent for the treatment of multiple myeloma (2, 3). Two independent laboratories have reported that bortezomib enhances melphalan response in vitro, implicating NF-κB inhibition due to blocked IκB degradation in this enhanced response (4, 5). Furthermore, bortezomib in combination with melphalan has shown encouraging activity in myeloma patients in a phase I/II trial (6). Also, in a separate, multicenter phase I/II study, the combination of bortezomib plus melphalan and prednisone was shown to be highly effective, even in patients with poor prognostic factors, and a phase III trial confirmed this finding (7–9). However, the mechanism by which bortezomib enhances melphalan response is unclear.

We first reported that the Fanconi anemia/BRCA (FA/BRCA) DNA damage repair pathway is a determinant of melphalan response and resistance (10, 11). Thirteen complementation groups have been identified from studies of Fanconi anemia patient cells, and all 13 genes (fanca, fancb, facce, fancd1, fancd2, fancf, fancg, fanci, fancj, fancl, fancm, and fancn) have been cloned (12–25). The key event in the activation of this pathway is the interdependent monoubiquitination of FANC2D2 and FANCL. Following monoubiquitination, the FANC2D2/I complex is targeted to sites of chromatin damage (22, 24, 26). Although the exact molecular function of the FA/BRCA pathway is poorly defined, its activation culminates in repair of DNA cross-links, UV-induced dimers, and double-strand breaks by homologous recombination and translesion synthesis (27, 28). Furthermore, this pathway has been shown to play an important role in the acquisition of drug resistance (10, 29, 30).

Our laboratory analyzed FA/BRCA pathway gene expression in myeloma cell lines and found that many FA/BRCA genes are overexpressed in melphalan-resistant myeloma cells when compared with drug-sensitive parental cells (10, 11). We also showed a causal relationship between levels of FANCf and melphalan response (10). Consequently, we reasoned that the transcriptional activation of the FA/BRCA pathway might allow for enhanced interstrand cross-link (ICL) repair, tumor progression, and the emergence of acquired drug resistance. NF-κB, a widely known transcriptional regulator of genes involved in cell survival, growth, angiogenesis, and metastasis, is constitutively activated in multiple myeloma (31, 32). Due to the central role of NF-κB in the pathogenesis of multiple myeloma and the relative abundance of putative NF-κB–binding sites on FA/BRCA promoter regions (Supplementary Fig. S1), we tested whether NF-κB could serve as a critical regulator of the FA/BRCA pathway in response to chronic melphalan-induced DNA damage.

In addition, we hypothesized that bortezomib enhances melphalan response by inhibiting expression and function of the FA/BRCA DNA damage repair pathway. This hypothesis is further substantiated by a recent report, which found that proteasome function is

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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required for FA/BRCA pathway activation (33). In the present study, we show that FANCD2 gene and protein expression are down-regulated in the presence of a nontoxic dose of bortezomib, and remain attenuated in the presence of melphalan, in drug-resistant cell lines. In these cells, formation of FANCD2 DNA repair foci was inhibited when cells were pretreated with bortezomib. Moreover, similar to the effect of bortezomib, loss of RelB and p50 induced a decrease in FANCD2 protein expression and sensitized 8226/LR5 cells to melphalan. Together, these results show that NF-κB functions as a regulator of FA/BRCA expression and suggest that bortezomib potentiates melphalan activity by inhibiting FA/BRCA pathway gene expression, reducing FANCD2 protein expression, and enhancing DNA damage likely via inhibition of DNA damage repair. These findings provide strong evidence for targeting the FA/BRCA pathway, and FANCD2 specifically, to enhance melphalan activity in myeloma patients.

Materials and Methods

Cell lines and materials. The 8226 and U266 human multiple myeloma cell lines were obtained from the American Type Culture Collection, and the corresponding melphalan-resistant cells (8226/LR5 and U266/LR6) were generated in our laboratory as described previously (34). All cell lines are routinely tested (every 3 months) for Mycoplasma contamination and κ/λ expression. For a complete list of materials and cell culture conditions, see Supplementary Information Text.

Electrophoretic mobility shift assays. Nuclear extracts were prepared from 8226 and U266 cells, and electrophoretic mobility shift assays (EMSA) were carried out as described previously (35). For gel shifts, nuclear extracts were incubated on ice with NF-κB–specific antibodies for 30 min before EMSA analysis. For a detailed protocol and a complete list of the EMSA probes used, see Supplementary Information Text.

Cell transfections. 8226 cells were seeded in complete medium at a concentration of 2 × 105/mL. After 24 h, 4 × 106 cells/sample were resuspended in 20 μL cytosine buffer containing 120 mmol/L KCl, 0.15 mmol/L CaCl2, 10 mmol/L K2HPO4/KH2PO4, 25 mmol/L HEPES, 2 mmol/L EGTA, 5 mmol/L MgCl2, 2 mmol/L ATP, and 5 mmol/L glutathione (pH 7.6), mixed with the indicated “ON-TARGET Plus” small interfering RNA (siRNA) duplexes (Dharmacon; see Supplementary Information Text) at a final concentration of 67 nmol/L, and electroporated at 140 V/1,200 μF. A similar transfection protocol was used for the overexpression of untagged, full-length FANCD2 using pIREs-neo-FANCD2 (exon 44 variant) plasmid DNA.

Immunoblotting. For detection of FANCD2, cell extracts were prepared by resuspending washed cell pellets in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1 mol/L NaCl, 0.1% NP-40, 1 mmol/L DTT] plus protease and phosphatase inhibitors. Samples were sonicated and incubated on ice for 30 min, and lysates were quantified with Bio-Rad reagent. After separation by SDS-PAGE, proteins were detected by standard immunoblotting.

Chromatin immunoprecipitation. 8226/S and 8226/LR5 cells were cross-linked in formaldehyde (20 × 10 s per sample) and lysed in SDS-rich buffer, and the resulting nuclear fractions were sonicated until the average chromatin size reached 200 to 1,000 bp (∼2−4, 10 s pulses/sample). The cell extracts were precleared with heat-inactivated, protein A–coated Staphylococcus aureus cells (PANSORBIN cells; Calbiochem) and immunoprecipitated with normal rabbit IgG or α-NF-κB/Rel–specific antibodies. After several washes, DNA/protein cross-links were reversed at 65°C, and a close-to-pure fraction of DNA was obtained with a ChIP-IT mini-column (Active Motif) and analyzed by quantitative PCR (qPCR; for details, see Supplementary Information Text).

Quantitative reverse transcription-PCR analysis. The RNeasy Micro kit (Qiagen) and SuperScript First Strand Synthesis kit (Invitrogen) were used for total RNA extraction and cDNA synthesis, respectively. Quantitative reverse transcription-PCR was carried out using either a customized microfluidic card or an Assay-on-Demand probe (Applied Biosystems; for details, see Supplementary Information Text).

Immunofluorescence analysis. Immunofluorescence techniques were used to analyze nuclear FANCD2 foci formation. Following fixation and permeabilization, cells were incubated with α-FANCD2 antibody for 2 h, washed in PBS, and visualized with Alexa Fluor 488 secondary antibody (Molecular Probes; Invitrogen) in the presence of 4,6-diamidino-2-phenylindole. For a detailed protocol, see Supplementary Information Text.

Diffusion Apoptosis Slide Halo Assay and Alkaline Comet Assay. The Diffusion Apoptosis Slide Halo Assay kit (Trevigen) was used to detect DNA fragmentation, an indicator of apoptosis. The Alkaline Comet Assay kit (Trevigen) was used to detect melphalan-induced DNA cross-links in 8226 cells as described previously (10). For details on both protocols, see Supplementary Information Text.

Isolation of primary myeloma cells. FA/BRCA pathway gene expression was analyzed in myeloma cells purified from four patients enrolled in an institutional review board–approved clinical trial for patients with primary refractory myeloma after obtaining informed patient consent. As per protocol, patients were treated with 1.3 mg/m2 bortezomib on days 1, 4, 8, and 11 every 3 weeks for two cycles. Following completion of the bortezomib cycles, patients underwent high-dose melphalan treatment immediately followed by one dose of bortezomib as conditioning regimen for tandem autologous peripheral blood stem cell transplants. Samples were collected and analyzed from three patients at the time of screening (baseline controls) and at 24 h after receiving the day 1 cycle 1 bortezomib dose. Samples analyzed from the fourth patient were collected at screening, after one dose of bortezomib, after two cycles of bortezomib, 3 months post-transplant, and at relapse. Plasma cells were isolated using a negative selection protocol, allowing for ≥95% purity. Briefly, 1 mL bone marrow aspirate was centrifuged in a Ficol-Paque Plus gradient (Amersham Biosciences), and a cytospin of the isolated cells was used to determine the initial purity of the plasma cell population. In parallel, 10 mL of the bone marrow sample were incubated with the Millennium Rosette Antibody Cocktail (Millennium Pharmaceuticals) for 20 min followed by Ficol extraction. A new cytospin was prepared from these cells to assess the efficiency of selection. Once myeloma cells were isolated, RNA was extracted, cDNA was synthesized, and qPCR analysis of FA/BRCA pathway genes was done as described above.

Statistical analysis. For a complete description of the statistical methods used in these studies, see Supplementary Information Text.

Results

Melphalan-induced NF-κB activity in myeloma cells. We reported previously that ICL accumulation in drug-sensitive 8226/S cells is ~2-fold higher than that of melphalan-resistant 8226/LR5 cells (10, 34). To assess directly the effect of acute melphalan exposure on NF-κB activation, we induced similar amounts of ICLs in 8226/S and 8226/LR5 cells by treatment with 25 and 50 μmol/L melphalan, respectively. Using EMSAs to measure DNA-bound NF-κB complexes, we found that 8226/LR5 cells exhibit a higher degree of basal and melphalan-induced DNA-binding activity relative to control cells (Fig. 1A). Moreover, melphalan stimulation elicited a peak of NF-κB activity after 30 min in both cell lines followed by a noticeable decrease in activity at 2 h post-treatment. These findings indicate that acute exposure to melphalan further increases the magnitude, but fails to accelerate the rate, of NF-κB activation in drug-resistant 8226/LR5 cells relative to drug-sensitive cells. Cellular extracts were immunoblotted with a phospho-specific IκB kinase (IKK) α/β antibody, and the results showed that IKKα was constitutively phosphorylated in 8226/LR5 cells but not in 8226/S cells (Fig. 1B). Interestingly, we found that IKKα phosphorylation was markedly reduced in 8226/LR5 cells after 1 h of melphalan treatment, whereas no IKKα activation was ever evident in 8226/S cells. A similar rate of IKKα phosphorylation was observed.
in drug-sensitive U266 and drug-resistant U266/LR6 cells after exposure to melphalan (Supplementary Fig. S2). Furthermore, we were unable to detect any basal or melphalan-induced phosphorylation of IKKα5 in either 8226 or U266 cells. These results show that chronic exposure of 8226 and U266 cells to melphalan engages the specific phosphorylation of IKKα leading to a concomitant increase in basal NF-κB DNA-binding activity.

**RelB/p50 regulates FANC D2 expression.** To determine if NF-κB can bind to the promoter region of FANC D2, nuclear extracts from 8226 cells were analyzed for NF-κB DNA-binding activity using FANC D2-specific NF-κB-binding sites as probes (denoted as I, II, III, and IV in Fig. 2A). NF-κB DNA-binding activity could only be detected with probe IV, and extracts from 8226/LR5 cells exhibited a substantial increase in FANC D2-bound NF-κB complexes relative to melphalan-sensitive cells (Fig. 2A). We next performed gel shift analyses and found that incubation of cell extracts with α-p50 or α-RelB antibodies resulted in a complete loss of probe IV-specific electrophoretic signals in 8226 cells (Fig. 2B). We also examined the effects of siRNAs targeted against NF-κB subunits on the binding activity of NF-κB toward FANC D2-probe IV. Knockdown of RelB or p50 reduced FANC D2-specific NF-κB activity in 8226/LR5 cells (Fig. 2C). Furthermore, direct immunoblotting of the cellular extracts used for the EMSA revealed a decrease in FANC D2 protein levels in RelB- and p50-depleted cells. Next, we subjected 8226/S and 8226/LR5 cells to a chromatin immunoprecipitation protocol designed to probe for binding between NF-κB subunits and the newly described NF-κB-binding site on the FANC D2 promoter (region IV, Fig. 2A). qPCR analyses of α-NF-κB immunoprecipitates with FANC D2-specific primers revealed that 8226/LR5 cells exhibited a higher degree of coimmunoprecipitation of region IV with RelB, p52, and p50 compared with melphalan-sensitive cells (Fig. 2D). In contrast, the association of this region of the FANC D2 promoter with p56 and c-Rel was found to be comparable in both cell lines. We conclude that the increase in FANC D2-specific NF-κB activity observed in melphalan-resistant cells is due to the enhanced binding of RelB and p50 to the FANC D2 promoter in these cells.

**BMS-345541 and bortezomib reduce FA/BRCA gene expression.** BMS-345541 is a selective inhibitor of the IKK complex and a putative antitumor agent (35). To examine the effect of loss of NF-κB function on FA/BRCA gene expression, we treated 8226 and U266 cells with 4 μmol/L BMS-345541 and monitored the levels of mRNA transcripts over time. Specifically, we analyzed eight Fanconi anemia genes (fancd1/brc a2, fanc a, fanc c, fanc d2, fanc e, fanc f, fanc g, and fanc l) as well as three DNA damage response genes (brca1, rad51, and rad51C) associated with this pathway. Importantly, at this drug concentration, BMS-345541 treatment caused a pronounced decrease in FANC D2 protein levels (Supplementary Fig. S3A). Expression of every gene analyzed (with the exception of fancl and rad51 in the 8226/S cells) significantly decreased in a time-dependent fashion during the first 4 to 8 h of BMS-345541 treatment (Fig. 3A; Supplementary Fig. S4 and B). However, after 8 h, exposure to BMS-345541 resulted in a gradual recovery in the expression of brca1, fanc, fanc e, and rad51C to baseline levels in both cell lines. Similarly, exposing U266 cells to BMS-345541 resulted in a striking decrease in the expression of all 13 FA/BRCA pathway genes after 8 h followed by a significant recovery in the expression of brc a2, fanc, fanc e, fanc f, fanc g, fanc l, fanc n, and fanc o in U266 and/or U266/LR6 cells at 12 h post-BMS-345541 treatment (Supplementary Figs. S5 and S6A-C). The biological significance of the latter event remains unclear, and results suggest that compensatory mechanisms in response to a protracted loss of NF-κB activity are involved in the regulation of the expression of these genes. Indeed, following stable inhibition of NF-κB, FA/BRCA pathway gene expression was inhibited to a lesser degree than the inhibition seen following transient inhibition (Supplementary Fig. S7), again suggesting that the activation of compensatory mechanisms is necessary following loss of NF-κB.

To further examine the role of NF-κB in melphalan resistance, we treated 8226 cells with increasing amounts of BMS-345541 for 96 h. Relative to sensitive cells, melphalan-resistant cells displayed a marked decrease in cell growth after BMS-345541 exposure (Supplementary Fig. S3B). These results suggest that NF-κB regulates FA/BRCA expression and that a sustained reduction in FA/BRCA gene expression is especially cytotoxic to melphalan-resistant myeloma cells.

To extend the BMS-345541 results to a more clinically relevant model, we analyzed FA/BRCA pathway gene expression following treatment with bortezomib, a known inhibitor of NF-κB that is presently used in the clinic for the treatment of myeloma (2, 4, 9). Using EMSAs, we first confirmed the work of others that low-dose bortezomib treatment with bortezomib, a known inhibitor of NF-κB in melphalan resistance, does indeed inhibit NF-κB activity (data not shown). Next, 8226/S and 8226/LR5 cells were treated with 10 μmol/L bortezomib for 2, 4, 8, and 24 h (Fig. 3B; Supplementary Fig. S8A and B). Bortezomib decreased expression of certain FA/BRCA pathway members in both cell lines in as little as 2 h, with maximal effect seen at 24 h.

**Figure 1.** Basal NF-κB DNA-binding activity is enhanced in melphalan-resistant 8226 cells. A, nuclear extracts were isolated from melphalan-treated 8226 cells at the indicated times and analyzed for NF-κB DNA-binding activity by EMSA using a canonical κB site as a probe. Binding to a NF-κB probe was used as a loading control. B, 8226/S and 8226/LR5 cells were treated with 25 and 50 μmol/L melphalan, respectively, to induce comparable amounts of ICLs between sensitive and resistant cell lines. Cells were harvested at the indicated times post-treatment and IKK phosphorylation was determined with a α-phospho-IKKα/IKKβ-specific antibody. Cellular extracts were then immunoblotted sequentially with α-IKKα, α-IKKβ, and α-β-actin antibodies.
Importantly, the downregulation of gene expression seen following treatment with bortezomib as well as with BMS-345541 is not due to a global inhibition of transcription, as certain genes analyzed remained unchanged following treatment (Supplementary Fig. S9). Of the FA/BRCA pathway–related genes that did change, fancd2 gene expression was most consistently and dramatically decreased by bortezomib in both drug-sensitive 8226/S and drug-resistant 8226/LR5 cell lines.

**Bortezomib potentiates melphalan cytotoxicity via inhibition of FANCD2.** As reported previously, pretreatment of myeloma cells with a nontoxic dose of bortezomib results in enhanced sensitivity to melphalan when compared with melphalan treatment alone (refs. 4, 5; Supplementary Fig. S10). To further characterize the effect of bortezomib on melphalan response, FANCD2 gene and protein expression was measured in the drug-resistant 8226/LR5 and U266/LR6 cell lines following 8 h pretreatment with either vehicle control or 3 nmol/L bortezomib and subsequent exposure to 25 μmol/L melphalan for 16 h. Results showed that bortezomib is a potent inhibitor of fancd2 gene expression in both drug-resistant cell lines even in the presence of melphalan (Fig. 4). Furthermore, bortezomib markedly reduced FANCD2 protein levels, in the presence or absence of melphalan, when compared with melphalan treatment alone (Fig. 4B).

Immunofluorescent microscopy techniques were used to analyze FANCD2 DNA repair foci formation, a hallmark of Fanconi anemia pathway activation. Melphalan-induced FANCD2 foci formation, as measured by percentage of cells with greater than five foci, was found to be greatly inhibited in cells pretreated with bortezomib (Fig. 4C). Notably, no difference in foci formation was observed between the control, bortezomib alone, and the...
Combination of bortezomib plus melphalan. Based on these results, we further explored the role of FANCD2 inhibition in the ability of bortezomib to enhance DNA damage induced by melphalan. Melphalan-induced DNA damage was found to be significantly enhanced in cells pretreated with 3 nmol/L bortezomib (Fig. 4D). Importantly, bortezomib treatment alone did not induce DNA damage when compared with control cells (data not shown). Moreover, similar differences in percentage of ICL formation are observed when comparing bortezomib plus melphalan and si-FANCD2 plus melphalan to their respective controls. Finally, previous reports have shown that FANCD2 monoubiquitination and foci formation is S phase specific (36). Consistent with these data, we also observed that bortezomib treatment decreases the percentage of cells in S phase (Supplementary Fig. S11), which may in part explain the decrease in total FANCD2 protein expression as well as FANCD2 foci formation following bortezomib treatment. Taken together, these results indicate that bortezomib enhances melphalan-induced DNA damage, leading ultimately to enhanced melphalan cytotoxicity, via the regulation of FANCD2 and the FA/BRCA pathway.

Melphalan-induced DNA fragmentation and ICL in RelB/p50-depleted cells. Next, we performed combination index analysis of BMS-345541 and bortezomib in 8226 and U266 cells. Results show that these compounds are additive in their cytotoxic effects (data not shown), suggesting that the effects of both agents are driven by inhibition of the NF-κB pathway. To further examine the role of NF-κB in the regulation of FANCD2 expression and function following treatment with melphalan, we determined whether RelB/p50 double knockdown was sufficient to sensitize 8226/LR5 cells to melphalan. Cells were treated with control or RelB and p50 siRNAs and then exposed to varying doses of melphalan for 24 h, and the amount of DNA fragmentation was determined with a Diffusion Apoptosis Slide Halo assay. Treatment of 8226/LR5 cells with RelB and p50 siRNAs significantly sensitized these cells to melphalan-induced cell death (Fig. 5A; Supplementary Fig. S12). Furthermore, relative to 8226/LR5 cells, RelB/p50-depleted cells...
displayed a dramatic increase in ICLs after exposure to melphalan, with levels of DNA damage significantly surpassing those observed in melphalan-sensitive cells (Fig. 5B; Supplementary Fig. S13). Moreover, the results presented in Fig. 5A and B were positively correlated at each dose of melphalan tested, suggesting that loss of cell viability is causally linked to melphalan-induced ICL (Supplementary Fig. S14). Importantly, consistent with the results obtained with RelB- and p50-depleted cells (Fig. 2C), RelB/p50 double knockdown caused a striking decrease in FANCD2 protein expression (Fig. 5C). Finally, reexpression of FANCD2 in the RelB/p50-depleted cells was able to restore melphalan resistance (Fig. 5C and D). Collectively, these results strongly suggest that RelB and p50 protect 8226/LR5 cells from melphalan-induced apoptosis, at least in part, by regulating expression of the DNA damage response protein FANCD2.

**Discussion**

We recently reported that the FA/BRCA DNA damage repair pathway is significantly involved in melphalan resistance in multiple myeloma cells (10, 11). It has also been reported that bortezomib enhances sensitivity to chemotherapeutic agents, including melphalan (4, 5). Based on these results, we hypothesized that bortezomib enhances melphalan cytotoxicity by inhibiting DNA repair associated with the FA/BRCA pathway. We found that bortezomib
treatment decreased expression of many FA/BRCA pathway genes in both melphalan-sensitive and melphalan-resistant cell lines, as well as in multiple myeloma patient specimens, suggesting that these genes may be coregulated. Furthermore, combination index analysis studies revealed additivity when myeloma cells were treated simultaneously with bortezomib and BMS-345541, two known inhibitors of the NF-κB pathway. The results suggest that both drugs exact their cytotoxic effects via inhibition of this pathway and implicate NF-κB as an upstream mediator of the FA/BRCA pathway.

We further report for the first time that NF-κB functions as a critical regulator of fancd2 expression. Interestingly, chronic exposure of 8226 cells to melphalan leads to an increase in basal NF-κB DNA-binding activity in these cells. RelB and p50 were identified as the subunits involved in this binding activity. Our chromatin immunoprecipitation results suggest that the level of NF-κB activity observed in melphalan-resistant cells is attributable to increased binding of RelB and p50 to the fancd2 promoter. As expected, RelB/p50-depleted 8226/LR5 cells displayed a dramatic decrease in FANC D2 protein expression, and acute melphalan treatment of these cells was accompanied by an increase in ICL and DNA fragmentation and the onset of apoptosis. Collectively, these findings point to alterations in FANC D2 expression and function as underlying events leading to increased melphalan sensitivity in RelB/p50-depleted cells. We postulate that genetic activation of the alternative NF-κB pathway allows for malignant plasma cells to gain independence from the rich milieu of growth factors and NF-κB-activating cytokines produced in the bone marrow microenvironment. Studies are under way to more precisely determine the mechanisms through which melphalan stimulation regulates IKKα phosphorylation and kinase activity in melphalan-resistant cells.

In addition to reducing gene expression, bortezomib inhibited FANC D2 protein expression and foci formation, even in the presence of melphalan, in two melphalan-resistant cell lines. Bortezomib was also found to enhance melphalan-induced DNA damage likely through inhibition of FANC D2 and thus abrogation of DNA repair. These results provide a mechanism by which to overcome or perhaps even prevent melphalan resistance. We speculate that treating patients first with bortezomib followed by melphalan may be necessary to capitalize on the ability of bortezomib to inhibit the FA/BRCA DNA damage response pathway. Conceptually, once this pathway is inhibited, melphalan may be more effective and result in more durable remissions.

Based on the present study, we propose that targeting the proteasome accentuates melphalan response by reducing FA/BRCA

![Figure 5](image-url)
gene expression and blocking activation of FANCD2, thereby inhibiting DNA damage repair following drug treatment. The authors recognize, however, that NF-κB and bortezomib affect expression of a large number of genes and proteins that may also contribute to enhanced melphalan cytotoxicity. For example, we have found and others have reported that bortezomib decreases the percentage of cells in S phase (37, 38), which may in part explain the decrease in FANCD2 protein expression and foci formation seen following bortezomib treatment (36). However, alterations in cell cycle progression do not address the transcriptional regulation underlying these changes in Fanconi anemia gene expression. Along these lines, an important conclusion from this study is that NF-κB functions as a regulator of FA/BRCA gene expression. Furthermore, Hoskins and colleagues have reported that Rb/E2F pathway family members are also involved in regulating Fanconi anemia gene expression (39). Predictably, the route to acquired melphalan resistance in myeloma cells involves the coordinated effects of myriad DNA damage–induced transcription factors, resulting in transient increases of FA/BRCA gene expression.

In summary, our findings provide insight into the mechanism by which bortezomib potentiates melphalan cytotoxicity. Based on our studies, we propose that the favorable therapeutic index of melphalan, and potentially other agents, in myeloma patients can be greatly enhanced by targeting the alternative NF-κB pathway with specific inhibitors of IKKε kinase activity. This work provides a target, the FA/BRCA DNA damage repair pathway, for enhancing chemotherapeutic response and reversing or possibly preventing drug resistance in myeloma patients.

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

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