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


1Cancer Biology Ph.D. Program, University of South Florida; Departments of 2Experimental Therapeutics and 3Immunology, 4Biostatistics Core, and 5Bone Marrow Transplantation Program, H. Lee Moffitt Cancer Center, Tampa, Florida

Abstract

The Fanconi anemia/BRCA (FA/BRCA) DNA damage repair pathway plays a pivotal role in the cellular response to repairative 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 subunits, RelB/p50, as transcriptional activators of the FA/BRCA pathway. Specifically, our findings point to constitutive phosphorylation of IκB kinase α and subsequent alterations in FANC D2 expression and function as underlying events leading to melphalan resistance in repeatedly exposed multiple myeloma cells. Inhibiting NF-κB by small interfering RNA, blocking the IκB kinase complex with BMS-345541, or using the proteasome inhibitor bortezomib drastically reduced FA/BRCA gene expression and FANC D2 protein expression in myeloma cells, resulting in diminished DNA damage repair and enhanced melphalan sensitivity. Importantly, we also found that bortezomib decreases FA/BRCA gene expression in multiple myeloma patients. These results show for the first time that NF-κB transcriptionally regulates the FA/BRCA pathway and provide evidence for targeting Fanconi anemia–mediated DNA repair to enhance chemotherapeutic response and circumvent drug resistance in myeloma patients.

Introduction

Multiple myeloma is a plasma cell malignancy characterized by initial therapeutic responses but rare cures secondary to the emergence of drug resistance (1). Melphalan, a DNA cross-linking agent, is one of the most commonly used drugs in the treatment of multiple myeloma. Because multiple myeloma patients relapse after initial therapy, discovering new treatment options that enhance response and prevent or overcome drug resistance are imperative for effectively eradicating this disease. Bortezomib is a reversible inhibitor of the 26S proteasome and a Food and Drug 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, fancc, 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 FANC D2 and FANCI. Following monoubiquitination, the FANC D2/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 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...
required for FA/BRCA pathway activation (33). In the present study, we show that FANCD2 gene and protein expression are downregulated in the presence of a nontoxic dose of bortezomib, and remain attenuated in the presence of melphan, 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 repressed 8226/LR5 cells to melphan. Together, these results show that NF-κB functions as a regulator of FA/BRCA pathway expression and suggest that bortezomib potentiates melphan 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 melphan 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 melphan-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 × 10^5/mL. After 24 h, 4 × 10^5 cells/sample were resuspended in 200 μL cytomix buffer [containing 120 mmol/L KCl, 0.15 mmol/L CaCl_2, 10 mmol/L K_2HPO_4/KH_2PO_4, 25 mmol/L HEPES, 2 mmol/L EGTA, 5 mmol/L MgCl_2, 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/0.15 mmol/L CaCl_2, 10 mmol/L K_2HPO_4/KH_2PO_4, 25 mmol/L HEPES, 2 mmol/L EGTA, 5 mmol/L MgCl_2, 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 mmol/L, and electroporated at 140 V/0.15 mmol/L CaCl_2, 10 mmol/L K_2HPO_4/KH_2PO_4, 25 mmol/L HEPES, 2 mmol/L EGTA, 5 mmol/L MgCl_2, 2 mmol/L ATP, and 5 mmol/L glutathione (pH 7.6). After several washes, DNA/protein cross-links were reversed at 65°C, and precipitated with normal rabbit IgG or specific antibodies for 30 min. These methods used in these studies, see Supplementary Information Text.

Results

Melphan-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 melphan-resistant 8226/LR5 cells (10, 34). To assess directly the effect of acute melphan 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 melphan, respectively. Using EMSAs to measure DNA-bound NF-κB complexes, we found that 8226/LR5 cells exhibit a higher degree of basal and melphan-induced DNA-binding activity relative to control cells (Fig. 1A). Moreover, melphan 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 melphan 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 Iκκα 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 melphan treatment, whereas no IKKα activation was ever evident in 8226/S cells. A similar rate of Iκκκ phosphorylation was observed in a 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 Slice Halo Assay and Alkaline Comet Assay. The Diffusion Apoptosis Slice Halo Assay kit (Trevigen) was used to detect DNA fragmentation, an indicator of apoptosis. The Alkaline Comet Assay kit (Trevigen) was used to detect melphan-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 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 melphan 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 first 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 Ficoll-Plaque Plus gradient (Amersham Biosciences), and a cytopsin of the isolated cells was used to determine the 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 Ficoll extraction. A new cytopsin 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.
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 melphan-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.

NF-κB can bind to the promoter region of FANC2, nuclear extracts from 8226 cells were analyzed for NF-κB DNA-binding activity using FANC2-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 FANC2-bound NF-κB complexes relative to melphan-sensitive cells (Fig. 2A). We next performed gel shift analyses and found that incubation of cell extracts with α50 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 FANC2-probe IV. Knockdown of RelB or p50 reduced FANC2-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 FANC2 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 FANC2 promoter (region IV, Fig. 2A). qPCR analyses of α-NF-κB immunoprecipitates with FANC2-specific primers revealed that 8226/LR5 cells exhibited a higher degree of coimmunoprecipitation of region IV with RelB, p52, and p50 compared with melphan-sensitive cells (Fig. 2D). In contrast, the association of this region of the FANC2 promoter with p65 and c-Rel was found to be comparable in both cell lines. We conclude that the increase in FANC2-specific NF-κB activity observed in melphan-resistant cells is due to the enhanced binding of RelB and p50 to the FANC2 promoter in these cells.

BMS-345541 and bortezomib reduce FA/BRC gene expression. BMS-345541 is a selective inhibitor of the IKK complex and a putative antitumor agent (35). To extend the BMS-345541 results to a more clinically relevant model, we analyzed FA/BRC 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 decreases expression of certain FA/BRC pathway members for 96 h. Relative to sensitive cells, melphan-resistant cells displayed a significant decrease in cell growth after BMS-345541 exposure (Supplementary Fig. S3B). These results suggest that NF-κB regulates FA/BRC gene expression and that a sustained reduction in FA/BRC gene expression is especially cytotoxic to melphan-resistant myeloma cells.

To further examine the role of NF-κB in melphan resistance, we treated 8226 cells with increasing amounts of BMS-345541 for 96 h. Relative to sensitive cells, melphan-resistant cells displayed a marked decrease in cell growth after BMS-345541 exposure (Supplementary Fig. S3B). These results suggest that NF-κB regulates FA/BRC gene expression and that a sustained reduction in FA/BRC gene expression is especially cytotoxic to melphan-resistant myeloma cells.
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 FANC2. 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, FANC2 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. 4A). Furthermore, bortezomib markedly reduced FANC2 protein levels, in the presence or absence of melphalan, when compared with melphalan treatment alone (Fig. 4B).

Immunofluorescent microscopy techniques were used to analyze FANC2 DNA repair foci formation, a hallmark of Fanconi anemia pathway activation. Melphalan-induced FANC2 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

Figure 2. RelB and p50 regulate FANCD2 expression in 8226 cells. A, nuclear extracts from 8226/S and 8226/LR5 cells were analyzed for NF-κB DNA-binding activity by EMSA using four FANC2-specific NF-κB probes (I, II, III, and IV). The specificity of each binding reaction was shown by competition with a 100-fold excess of unlabeled (cold) NF-κB canonical probe. B, experiment was done as described in A, except that nuclear extracts were incubated with the indicated antibodies for 30 min before EMSA analysis. Binding to FANC2-probe IV was used as a measure of NF-κB activity on FANC2 promoter region. C, 8226/LR5 cells were transfected with the indicated siRNAs and examined by EMSA using FANC2-probe IV. Binding to a NF-1 probe was used as a loading control. Bottom, expression levels of FANC2, p65, RelB, c-Rel, p50, p52, and β-actin at 48 h post-transfection. Expression levels of TRAF2 and FLIPL (two positive controls) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; negative control) are also shown. D, binding of NF-κB to the FANC2 promoter is enhanced in 8226 melphalan-resistant cells as determined by chromatin immunoprecipitation analysis. DNA samples were subjected to qPCR using glyceraldehyde-3-phosphate dehydrogenase and FANC2-specific primer pairs, schematically represented as Control and IV. Results were obtained from three independent trials, and fold changes in gene expression were normalized to input samples.
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

![Figure 3. BMS-345541 and bortezomib downregulate FA/BRCA pathway mRNA expression. A, 8226/S and 8226/LR5 cells were treated with 4 μmol/L BMS-345541 and harvested at the indicated times. FA/BRCA gene expression was determined in quadruplicate samples by qPCR using a customized microfluidic card. Results depict relative change normalized to vehicle control (DMSO) samples. Statistical analysis results can be seen in Supplementary Fig. S4. B, 8226/S and 8226/LR5 cells were treated with 10 nmol/L bortezomib and harvested at indicated times. FA/BRCA gene expression was determined as described in A. Statistical analysis results can be seen in Supplementary Fig. S8.](image-url)
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.

**Bortezomib reduces FA/BRCA pathway gene expression in patient specimens.** To extend the results obtained in *vitro* to the clinic, we analyzed FA/BRCA pathway gene expression in purified plasma cells from three myeloma patients before and after treatment with bortezomib. The results revealed that, in all three patients, a single dose of bortezomib reduced FA/BRCA gene expression when compared with baseline (screening) levels (Fig. 6A). Furthermore, we were able to analyze myeloma cells collected from a fourth patient before therapy, following one dose of bortezomib, two cycles of bortezomib, 3 months after high-dose melphalan and stem cell transplant, and at time of relapse. Bortezomib administration moderately inhibited FA/BRCA pathway gene expression in this patient, and FA/BRCA genes were highly overexpressed 3 months after high-dose melphalan compared with baseline; the patient clinically relapsed only 5 months after completion of therapy (Fig. 6B). Importantly, these preliminary results fully support our hypothesis that bortezomib treatment leads to the inhibition of FA/BRCA gene expression in multiple myeloma patients.

**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 facncd2 promoter. As expected, RelB/p50-depleted 8226/LR5 cells displayed a dramatic decrease in FANCD2 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 FANCD2 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 FANCD2 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 FANCD2 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. Loss of RelB and p50 re-sensitizes 8226/LR5 cells to melphalan treatment and reduces FANCD2 expression. A, 8226/LR5 cells were treated with the indicated siRNAs for 48 h and stimulated with 25 to 100 μmol/L melphalan or vehicle control for 24 h, and the relative amount of DNA fragmentation was determined with a Diffusion Apoptosis Slide Halo Assay. Wild-type 8226/S and 8226/LR5 cells were used as reference samples. B, at 48 h post-transfection, cells were exposed to 25 to 100 μmol/L melphalan for 2 h and damaged with 9 Gy ionizing radiation, and ICLs were evaluated with the Alkaline Comet Assay kit. Wild-type 8226/S and 8226/LR5 cells were used as reference samples. C, representative immunoblots from 8226/LR5 cells transfected with siControl (control) or RelB and p50 siRNAs as well as from cells transfected with RelB/p50 siRNAs plus full-length FANCD2 (pIRES-FANCD2). D, 8226/LR5 cells were treated with the indicated siRNAs, and after 24 h, cells were transfected with empty vector or untagged, full-length FANCD2 (pIRES-FANCD2). At 48 h post-siRNA transfection, cells were stimulated for 24 h with either vehicle control (VC) or 50 μmol/L melphalan, and the relative amount of DNA fragmentation was determined as described in A. Mean ± SE from three independent trials.
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 IKKα 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.

**Acknowledgments**

Received 7/13/09; revised 9/21/09; accepted 10/9/09; published OnlineFirst 11/24/09.

**Grant support:** NIH grants CA 077859-09 and CA2 P30 CA076292-10, Multiple Myeloma Research Foundation (15-06), and Molecular Biology, Flow Cytometry, and Analytic Microscopy Core Facilities at the H. Lee Moffitt Cancer Center and Research Institute. NIH-National Cancer Institute grant R01 CA77859: drug resistance in multiple myeloma.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Joel Turner and Paula Oliveira for technical assistance; Rachael Evey for help with article preparation; Drs. Mark Meads, Zhi-Wei Li, Mohamed Hussein, and Álvaro Monteiro for helpful discussion; and Dr. Markus Grompe (Oregon Health & Science University) for generously providing the pRES-neo-FANCD2 (exon 44 variant) plasmid.
Targeting the Fanconi Anemia/BRCA Pathway Circumvents Drug Resistance in Multiple Myeloma

Danielle N. Yarde, Vasco Oliveira, Linda Mathews, et al.

Cancer Res  Published OnlineFirst November 24, 2009.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-2616

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/11/24/0008-5472.CAN-09-2616.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.