Research Article

The Novel, Proteasome-Independent NF-κB Inhibitor V1810 Induces Apoptosis and Cell Cycle Arrest in Multiple Myeloma and Overcomes NF-κB–Mediated Drug Resistance

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Abstract

Evidence is increasing that aberrant NF-κB activation is crucial for multiple myeloma pathophysiology and is a promising target for new antimyeloma therapies. In this study, we assessed the in vitro antimyeloma activity of the novel NF-κB inhibitor V1810. Pharmacokinetics and toxicity were studied in vivo. In mice, V1810 plasma concentrations of 10 μmol/L can be reached without relevant toxicity. At this concentration, V1810 potently induces apoptosis in all four multiple myeloma cell lines assessed (IC₅₀ = 5–12 μmol/L) as well as in primary multiple myeloma cells (IC₅₀ = 5–40 μmol/L). Apoptosis induced by V1810 is associated with proteasome-independent inhibition of NF-κB signaling (41% relative reduction), downregulation of Mcl-1, and caspase 3 cleavage. In OPM2, U266, and RPMI-8226 cells, induction of apoptosis is accompanied by cell cycle arrest. Western blots revealed downregulation of Cdk4 as well as cyclin D3 (U266) or cyclin D2 (OPM2, NCI-H929, RPMI-8226), but not cyclin D3. Consistently, retinoblastoma protein was found to be hypophosphorylated. Furthermore, V1810 reverses NF-κB activation induced by the genotoxic drugs melphalan and doxorubicin. V1810 and melphalan synergistically decrease multiple myeloma cell viability. Taken together, the novel, proteasome-independent NF-κB inhibitor V1810 induces apoptosis and cell cycle arrest in multiple myeloma cells at a concentration range that can be achieved in vivo. Moreover, V1810 reverses NF-κB activation by alkylating drugs and overcomes NF-κB–mediated resistance to melphalan. Mol Cancer Ther; 9(2) February 2010; DOI: 10.1158/1535-7163.MCT-09-0645

Introduction

Multiple myeloma is the second most common hematologic malignancy. The disease is characterized by the accumulation of mature antibody-producing plasma cells in the bone marrow. Despite recent advances in the therapy of multiple myeloma and the emergence of novel agents such as bortezomib, thalidomide, and lenalidomide, the disease remains incurable. Patients eventually become resistant to chemotherapy and die of disease progression or therapy complications. Novel drugs are urgently needed to overcome drug resistance.

Constitutive NF-κB activity is a common characteristic of both primary multiple myeloma cells and multiple myeloma cell lines (1). Recently, molecular mechanisms of aberrant NF-κB activity in multiple myeloma have been elucidated. The genetic events underlying constitutive NF-κB activity in multiple myeloma include gain of function mutations of NF-κB activators such as CD40, NF-κB–inducing kinase (NIK), and NF-κB itself, as well as mutations inactivating NF-κB repressors, most commonly TRAF3 (2–4). NF-κB defines a family of transcription factor dimers consisting of Rel family proteins p65 (Rel A), Rel B, c-Rel, p50/p105, and p52/p100. NF-κB is inactive when bound to NF-κB inhibitory proteins (IκB) in the cytoplasm. Upon stimulation by inflammatory cytokines, IκB is phosphorylated by IκB kinases (IKK) and cleaved by the 26S proteasome, thus releasing NF-κB. NF-κB subsequently translocates into the nucleus and induces transcription of target genes (5). The significance of the NF-κB pathway for multiple myeloma pathophysiology is well established and characterized. NF-κB promotes growth, survival, and drug resistance in multiple myeloma cells (6). Moreover, NF-κB upregulates adhesion molecules and promotes cytokine secretion in both multiple myeloma cells and bone marrow stromal cells, thus contributing to cell adhesion–mediated drug resistance (7–9). Accordingly, inhibitors of IKKβ inhibit multiple myeloma cell growth (10–12) and first NF-κB inhibitors have been shown to induce apoptosis in multiple myeloma cells (6, 13). Moreover, the proteasome inhibitor bortezomib has shown not only antimyeloma activity in vitro and in vivo (14), including poor-prognosis subtypes of multiple myeloma (15), but also clinical efficacy in multiple myeloma patients (16) which led to approval by the Food and Drug Administration in 2003 (17). Bortezomib’s antimyeloma activity is likely to be in large part due to its repression of NF-κB signaling (18–20).
We and others have shown previously that genotoxic drugs such as melphalan and doxorubicin lead to NF-κB activation in multiple myeloma cells (21, 22). This suggests that upregulation of NF-κB is a mechanism by which malignant cells escape chemotherapy-induced apoptosis. Bortezomib has been shown to reverse drug-induced NF-κB activation, thus synergistically decreasing myeloma cell viability (21, 23). Similarly, inhibitors of IKK have been previously shown to have synergistic antymyeloma activity with genotoxic drugs (12). Therefore, NF-κB inhibition is a promising strategy to sensitize myeloma cells to chemotherapy and to be incorporated in multiagent chemotherapies.

It has been shown recently that in many primary multiple myeloma cells, NF-κB is activated by proteasome inhibitor–resistant pathways (24). These findings might explain why a significant percentage of patients show primary resistance or become refractory to bortezomib. Hence, new NF-κB inhibitors with a proteasome-independent mechanism of action are needed to overcome drug resistance, particularly in those patients refractory to bortezomib treatment.

In 2007, a novel class of potent NF-κB inhibitors was identified in a NF-κB reporter gene assay using secreted alkaline phosphatase as a reporter gene which was stably transfected in A549 cells under control of a NF-κB specific promoter (25). In preliminary experiments with several compounds of this class of NF-κB inhibitors we selected V1810 for displaying particular cytotoxic selectivity for multiple myeloma cells and carried out the present study to assess the antymyeloma activity of V1810.

**Materials and Methods**

**Cells**

NCI-H929, U266, RPMI-8226, and OPM-2 cell lines were obtained from the American Type Culture Collection, grown in RPMI 1640 medium (Boehringer) containing 10% heat-inactivated FCS (Boehringer) in a humidified atmosphere (37°C; 5% CO2) and seeded at a concentration of 1 × 10⁵ cells/mL. After informed consent was provided by patients, mononuclear cells were isolated from healthy donors after informed consent was obtained. The ethics committee of the University of Munich approved the study.

**Reagents**

V1810 was supplied by 4SC AG. Melphalan and doxorubicin were purchased from Calbiochem, bortezomib from Millenium Pharmaceuticals, and the cell reagent WST-1 from Roche. Annexin V-FITC was acquired from Pharmingen (BD Biosciences) and propidium iodide (PI) from Calbiochem. Goat polyclonal antibodies for actin, rabbit polyclonal antibodies for p-Rb were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies for p-Rb were obtained from Cell Signaling. A polyclonal antibody for Bcl-2 was purchased from BD Biosciences. Secondary, horseradish peroxidase–coupled polyclonal rabbit anti-goat and polyclonal swine anti-rabbit antibodies were purchased from DakoCytomation. A secondary horseradish peroxidase–coupled polyclonal goat anti-mouse antibody was obtained from Santa Cruz Biotechnology.

**In vivo Pharmacokinetics**

Female, immunocompetent NMRI mice were obtained from Janvier and treated with i.p. or i.v. injection of V1810. For the i.v. solution, V1810 was diluted to a final concentration of 1 mg/mL in PBS containing 20% cremophor. For the i.p. vehicle solution PBS containing 10% cremophor was used. Application volumes were determined based on body weight. Plasma levels of V1810 were quantified using liquid chromatography-mass spectrometry.

**In vivo Toxicity Profiling**

Female BalbC mice were treated with i.v. injections of 10, 20, or 30 mg/kg V1810 daily for 4 d. Mice treated with NaCl or vehicle solution (containing 10% DMSO and 20% cremophor RH 40) served as control groups (n = 4 for NaCl group, n = 7 for vehicle solution and V1810 10 mg/kg, n = 8 for V1810 20 mg/kg and 30 mg/kg). Body weight was monitored every morning. Clinical chemistry was done on day 5, 24 h after the last injection. Behavior was assessed by a standardized score on days 1, 2, and 4. Liver and spleen weight were measured post mortem on day 5.

**Analysis of Cell Cycle**

Cells were fixed overnight with 70% ice-cold ethanol. Cells were resuspended in 1 mL of PBS containing 40 μg/mL PI and 500 U/mL RNase A. Following incubation for 30 min in the dark at room temperature, cells were analyzed on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter) within 30 min.

**Analysis of Cell Death and Apoptosis by Flow Cytometry**

Myeloma cells were seeded at a concentration of 1 × 10⁵ cells/mL. After incubation, multiple myeloma cells were stained with FITC-conjugated Annexin V and PI. Cells were resuspended in binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl2). Of this cell suspension, 100 μL were incubated with 5 μL Annexin V-FITC and 10 μL of 50 μg/mL PI for 15 min at room temperature in the dark. Cells were analyzed on a Coulter EPICS XL-MCL flow cytometer using the System II software. The PI fluorescence signal peak versus the integral was used to discriminate G2-M cells from G0/G1 doublets.

**Immunoblot Analysis**

Cells were lysed in a buffer containing 10 mmol/L Tris-HCl (pH 7.6), 137 mmol/L NaCl, 1 mmol/L Na3VO4, 10 mmol/L NaF, 10 mmol/L EDTA, 1% (w/v) Igepal CA-630 (NP-40)
with the addition of 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonylfluoride. Protein concentration was adjusted using a colorimetric assay (Bio-Rad Protein Assay, Bio-Rad). Proteins were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore). Membranes were blocked with PBS containing 5% dried milk and 0.05% Tween-20, incubated with appropriate primary and secondary antibodies and visualized by autoradiography using the ECL Western blotting detection system (GE Healthcare).

**WST-1 Cell Viability Assay**

For quantification of viable cells in suspension a WST-1 viability assay protocol was used as recommended by the manufacturer (Roche). Briefly, 4 × 10^3 cells/100 μL were incubated with the test compounds for 48 h. For the last 2 h of culture, cells were incubated with 10 μL of WST-1 reagent. Absorbance at 450 nm was measured using a microplate ELISA reader to detect metabolically intact cells (reference wavelength: 680 nm).

**NoShift NF-κB Binding Assay**

After incubation, cells were washed with PBS and nuclear extracts were prepared by using the NucBuster Protein Extraction Kit (Novagen, Merck Biosciences). The binding affinity of multiple myeloma cell nuclear extracts to NF-κB (p65) DNA was determined with the NoShift Transcriptional Factor Assay Kit (Novagen, Merck Biosciences) according to the manufacturer’s instructions.

**Proteasome Redistribution Assay**

The proteasome redistribution assay was done by Fisher BioImage ApS (Denmark). The proteasome assay monitors ubiquitin/proteasome-dependent proteolysis of a green fluorescent protein (GFP)-based substrate. The GFP substrate consists of a mutated uncleavable ubiquitin moiety [Ubi(G76V)] fused to GFP resulting in constitutive degradation of the protein. U2OS ps 2042 [Ubi (G76V)-GFP] cells were treated with V1810. DMSO concentration in assay wells did not exceed 0.25%. The irreversible proteasome inhibitor MG-132 was used as reference compound. Accumulation of Ubi(G76V)-EGFP in the nucleus was imaged by fluorescence microscopy.

**Proteasome-Glo Assay**

Proteasome-Glo Chymotrypsin-Like Cell-Based Assay (Promega) was done according to the manufacturer’s instructions. Briefly, rat PBMCs (100,000 cells/well) were plated in 90 μL/well. The cells were incubated with V1810 for 2 h at 37°C, 5% CO2. DMSO concentration in the wells did not exceed 0.1%. The proteasome inhibitor lactacystin served as a reference compound. A total of 100 μL/well of each Proteasome-Glo Cell-Based Reagent was added and luminescence was measured with the Tecan Ultra Reader (1,000 ms) after 15 min.

**Statistics**

The Mann-Whitney-U-test was used to determine the significance of differences between two experimental groups; P < 0.05 was considered statistically significant. To assess whether combination treatments resulted in additive or synergistic effects, the combination index (CI) was calculated by using the Calcusyn software. CI = 1.1–0.9 was regarded as additive, CI < 0.9 as synergistic.

**Results**

**V1810 Shows Favorable Pharmacokinetics and Tolerable Toxicity in Mice**

V1810, with chemical structure 2-(1-Thieno[3,2-d]pyrimidin-4-yl-piperidin-4-yl)-thiazole-4-carboxylic acid (6-benzoyl-1H-benzoimidazol-2-yl)-amide (Fig. 1A),

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Figure 1. Chemical structure, pharmacokinetics, and toxicity profile of V1810. A, the chemical structure of V1810 is 2-(1-Thieno[3,2-d]pyrimidin-4-yl-piperidin-4-yl)-thiazole-4-carboxylic acid (6-benzoyl-1H-benzoimidazol-2-yl)-amide. Its molecular weight is 565.68 g/mol. B, female NMRI mice were treated with i.p. injections of 15 mg/kg V1810 once daily. Plasma concentrations of V1810 were measured at 15 and 30 min, and 1, 2, 4, and 6 h after injection. C, female NMRI mice were treated with i.v. injections of 5 mg/kg V1810 once daily. Plasma concentrations of V1810 were measured at 15 and 30 min, and 1, 2, and 4 h after injection.
is a small molecule (molecular weight 565.68 g/mol) that was identified as a potent NF-κB signaling inhibitor (25). To assess the pharmacokinetics of V1810, female NMRI mice were treated with i.p. injection of V1810. In this setting, 15 mg/kg i.p. once daily was sufficient to reach plasma concentration higher than IC50 of antimyeloma activity as shown below [maximum drug concentration (Cmax) was 7,367 ng/mL; Fig. 1B]. The terminal half-life (t1/2) was found to be 54 minutes and the area under the curve [AUC0-∞] was 13,480 ng/mL×h. At this dosage, V1810 did not induce any apparent side effects or death in NMRI mice. Similarly, iv. injections of 5 mg/kg daily yielded concentration comparable with the concentrations used in this in vitro study (Cmax = 4,531 ng/mL = 8 μmol/L; Fig. 1C). Under i.v. treatment with 5 mg/kg, no apparent side effects were observed.

To further evaluate toxicity profile, BalbC-mice were treated with i.v. injections of 10, 20, or 30 mg/kg V1810 daily for 5 days. Mice treated with NaCl or vehicle solution (containing 10% DMSO and 20% cremophor RH 40) served as control groups (n = 4 for NaCl group, n = 7 for vehicle solution and V1810 10 mg/kg, n = 8 for V1810 20 mg/kg and 30 mg/kg; Table 1). There were no significant changes in body weight in either group. Dose-dependent behavioral changes were observed but disappeared after 2 days of treatment. Chemistry did not reveal any significant changes in levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase, urea, bilirubine, creatinine, or hemoglobin after 5 days of V1810 treatment. Liver weight was increased only in the high-dose treatment groups (20 and 30 mg/kg) compared with mice treated with vehicle solution. One and two spontaneous deaths respectively were observed in the high-dose treatment groups (20 and 30 mg/kg). Taken together, 10 mg/kg i.v. daily is a safely tolerable dose of V1810. Plasma concentrations of about 10 μmol/L can be easily achieved in animals without relevant toxicity. Phase I studies for V1810 in human are urgently needed. So far, based upon our experiments, a dose range of about 10 μmol/L was considered clinically relevant and was subsequently used for the in vitro studies.

**V1810 Potently Induces Apoptosis in Multiple Myeloma Cells**

V1810 dose-dependently induced cell death in all four multiple myeloma cell lines assessed (OPM2, U266, NCI-H929, RPMI-8226) with IC50 ranging from 5 to 12 μmol/L (Fig. 2A and C). Cell death occurred in a time-dependent manner, with cell viability decreasing to as little as 10% to 25% within 72 hours (Fig. 2D). Importantly, the viability of control PBMCs remained almost unaffected even at very high concentrations (89% viability at 100 μmol/L; 10 and 30 μmol/L shown in Fig. 2C). Marked activation of caspase 3 occurred less than 12 hours after V1810 treatment (Fig. 2B). V1810 also induced cell death in primary multiple myeloma cells obtained from patients. In these primary multiple myeloma cells, 10 μmol/L of V1810 decreased cell viability by up to 60% (IC50 values range from 5 to 40 μmol/L; Fig. 2C). The patients enrolled in this study included newly diagnosed multiple myeloma (patient 3), as well as relapses after two cycles of high-dose melphalan followed by autologous stem cell transplants (patients 2 and 4) and two patients relapsing after miscellaneous chemotherapies who had been clearly refractory to bortezomib clinically (patients 1 and 5).

**V1810-Induced Apoptosis Is Associated with Proteasome-Independent Inhibition of NF-κB**

To assess the molecular mechanisms underlying V1810-induced apoptosis we examined the effects on NF-κB p65 and proteasome activity. V1810 strongly inhibits

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<th>Table 1. V1810 shows tolerable toxicity at effective concentrations</th>
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**NOTE:** BalbC-mice were treated with i.v. injections of 10, 20, or 30 mg/kg V1810 daily. Control groups were treated with NaCl and vehicle solution (containing 10% DMSO and 20% cremophor RH40), respectively. Abbreviations: ALT/GPT, alanine aminotransferase; AST/GOT, aspartate aminotransferase; AP, alkaline phosphatase.
constitutive NF-κB binding activity at 10 μmol/L with a 47% relative reduction at 2 hours ($P = 0.02$; Fig. 3A) persisting at 8 hours (41% inhibition, $P = 0.001$; Fig. 3A). This inhibition of NF-κB p65 activity is considerably stronger than found for a similarly toxic concentration (4 nmol/L) of bortezomib (14% inhibition at 8 hours; data not shown). In contrast, proteasome redistribution assay did not show any inhibition of proteasome activity at concentrations of up to 31 μmol/L (Fig. 3B). We confirmed this result by doing the Proteasome Glo Assay. Again, there was no relevant inhibition of proteasome activity. At 17 μmol/L, proteasome activity was still 98% of untreated control (Fig. 3C). This suggests that V1810 inhibits NF-κB by a proteasome-independent mechanism. To assess which step of the NF-κB signaling pathway is inhibited by V1810, we carried out Western blotting experiments for IκBα and p-IκBα (phosphorylated on IKK phosphorylation site Ser$^{32}$). These experiments clearly show that V1810 does not act by inhibiting phosphorylation of IκB and the consequent increase in total IκB levels. Rather, total IκB levels slightly decreased after V1810 treatment, which is likely to be a secondary effect due to NF-κB inhibition as
κB itself is a NF-κB target gene (26). This indicates that V1810 does not act by inhibiting IKK or NF-κB signaling upstream of IKK, but directly inhibits NF-κB. We further examined modulation of antiapoptotic and proapoptotic signaling pathways as previously reported to be affected by NF-κB inhibition in multiple myeloma. Bcl-2 has been found to be NF-κB dependent in many models (27–29), and the NF-κB inhibitor SN50 has been found to decrease levels of both Bcl-2 and cIAP-1 (6). We did not see changes in levels of Bcl-2 and cIAP1 (Fig. 3D). By contrast, Mcl-1 was found to be clearly downregulated (Fig. 3D). These data suggest that V1810 induces apoptosis in multiple myeloma cells by proteasome-independent NF-κB inhibition and consequent downregulation of specific antiapoptotic proteins such as Mcl-1, resulting in caspase activation.

**V1810 Induces Cell Cycle Arrest**

Because NF-κB is known to be involved in cell cycle control, we next examined the effect of V1810 on cell cycle progression. Clear cell cycle arrest was observed in three of four cell lines (OPM2 and U266, G0-G1 arrest; RPMI-8226, G2 arrest; Fig. 4A and B). In OPM2 cells, the G0/G1 fraction sharply increased from 35.8% to 52.9% under V1810 treatment; a similar G0/G1 accumulation was seen in U266 (increase from 43.6% to 61.4%). In contrast, RPMI-8226 cells displayed an increase of cells in G2 phase from 20.7% to 50.3%. Inhibition of cell cycle progression occurred simultaneously to apoptosis. In NCI-H929 cells, massive DNA cleavage caused by V1810 hampered assessment of cell cycle. When treated with low-toxic concentrations of V1810, no cell cycle arrest was observed.

**Cell Cycle Arrest Is Mediated by Downregulation of Cdk4 and Cyclin D1/2 Resulting in Hypophosphorylation of Retinoblastoma Protein**

To further investigate the molecular mechanisms underlying cell cycle inhibition by V1810 we studied the effect on key proteins involved in cell cycle regulation. We focused on OPM2 cells as a model of the G1 arrest that was seen in two of four cell lines. We found that cyclin D2 was downregulated in OPM2 cells very early after
Figure 4. Cell cycle modulation by V1810. A and B, OPM2, U266, and RPMI-8226 cells were treated with 10 μmol/L of V1810 for 48 h. NCI-H929 cells were treated with 6 μmol/L V1810 for 48 h, because at 10 μmol/L strong DNA cleavage in NCI-H929 cells hampered cell cycle analysis. Cell cycle analysis was done by flow cytometry after PI staining. Representative original data for OPM2 and RPMI are shown in Fig. 4A. Mean values are shown in Fig. 4B. C, time-dependent modulation of cell cycle regulators by V1810. OPM2 cells were treated with V1810 (10 μmol/L) for 0, 4, 12, and 24 h. Cell lysates were subjected to Western blot analysis. D, modulation of D type cyclins by V1810 in various multiple myeloma cell lines. NCI-H929, OPM2, U266, and RPMI-8226 multiple myeloma cells were incubated with 10 μmol/L of V1810 for 4 h. For RPMI-8226, the experiment was also done with a 24-h incubation period. Cell lysates were subjected to Western blot analysis.
V1810 treatment (Fig. 4C). There was also a decrease in levels of Cdk4, but not Cdk6. D-type cyclins (consisting of cyclins D1, D2, and D3) are cofactors of cyclin-dependent kinases 4 and 6 (Cdk4 and Cdk6). When bound to cyclin D, Cdk4 and Cdk6 are active and phosphorylate target proteins, with Rb being their primary substrate (30). Therefore, we examined the effects of V1810 treatment on levels of Rb phosphorylated on Cdk4/6-specific sites Ser780 and Ser807/811 (p-Rb). Concordant with Cdk4 and cyclin D2 downregulation, we found that levels of p-Rb time-dependently declined and were hardly detectable after 24 hours of V1810 treatment (Fig. 4C). There was a slight decrease in total levels of Rb protein, but this was not strong enough to account for the decrease in p-Rb level.

Expression of D-type cyclins varies between multiple myeloma cell lines. We therefore examined cyclin D expression and modulation in all four cell lines. Interestingly, cyclin D1/D2 downregulation was a common early event in all tested multiple myeloma cell lines (Fig. 4D). In OPM2, NCI-H929, and RPMI-8226 cyclin D2 was downregulated by V1810, whereas no protein levels of cyclin D1 were detectable. In U266, by contrast, only cyclin D1 was expressed. Here, V1810 led to a decrease in cyclin D1 level. Cyclin D3 expression was seen in NCI-H929 and OPM2 but not in U266 and RPMI-8226 cells. In sharp contrast to cyclin D1/D2 modulation, V1810 did not affect cyclin D3 levels. In all cell lines except RPMI-8226 downregulation of cyclin D1/D2 occurred within the first 4 hours of V1810 treatment (Fig. 4D).

We further investigated protein levels of cyclin-dependent kinase inhibitors (CKI) to rule out the possibility that Rb hypophosphorylation and cell cycle arrest were due to enforced expression of CKIs. CKIs include two distinct families. Members of the INK4 family (p16INK4a, p15INK4b, p18INK4c, p19INK4d) exclusively bind to D-type cyclin-dependent kinases Cdk4 and Cdk6. Members of the CIP/KIP family (p21CIP1/WAF1, p27KIP1, p57KIP2), in contrast, are universal inhibitors of all Cdk5 (30). We found that neither levels of INK4 proteins p16INK4a and p18INK4c nor p21CIP1/WAF1 showed modulation by V1810 (Fig. 4C). Levels of p27KIP1 decreased with V1810 treatment, whereas there were no detectable levels of p15INK4b, p19INK4d, and p57KIP2. These results suggest that cell cycle inhibition is specifically mediated by downregulation of Cdk4 and cyclin D1/D2 and consequent hypophosphorylation of Rb.

V1810 Reverses Activation of NF-κB Induced by Genotoxic Drugs

So far we have shown that V1810 potently induces apoptosis and downregulates Cdk4 and cyclin D1/D2, thus inducing cell cycle arrest in myeloma cells. Given that cyclin D1 and D2 are known to be under NF-κB control (31), we hypothesized that NF-κB inhibition was the common mechanism by which V1810 induces both apoptosis and cell cycle arrest. As a next step, we examined whether V1810 could also inhibit NF-κB activation induced by genotoxic drugs common in the treatment of multiple myeloma. The most important alkylating agent in the treatment of multiple myeloma is melphalan. The first-line treatment of younger patients is high-dose melphalan with autologous stem cell transplantation, whereas the primary treatment of elderly or medically unfit patients consists of drug combinations of melphalan and steroids with either bortezomib or thalidomide. NF-κB activity notably increased after treatment with melphalan (2.5-fold, P = 0.001; Fig. 5A). V1810 was able to completely reverse this effect (54.4% versus

**Figure 5.** Reversal of NF-κB–mediated drug resistance by V1810. A, V1810 reverses drug-induced activation of NF-κB. OPM2 cells were treated with melphalan (10 μmol/L) in the presence or absence of V1810 for 8 h. Melphalan was added after a 90-min preincubation with V1810. Nuclear extracts were prepared and NF-κB binding activity was quantified using the NoShift Transcription Factor Assay. Experiments were done at least in triplicate, and mean values and SD are shown. B, synergistic antimyeloma effects of V1810 and melphalan. OPM2 cells were treated with melphalan (6 μmol/L or 10 μmol/L) in the absence or presence of V1810 (6 μmol/L or 10 μmol/L) in the presence or absence of V1810 (6 μmol/L or 10 μmol/L). Melphalan was added after a 90-min preincubation with V1810. Assay plates were incubated for 48 h. The WST-1 reagent was added for the last 2 h of incubation. Absorbance was measured at 450 nm. Mean values and SD are shown.
250.4%, \( P = 0.001 \); Fig. 5A). Similar effects were observed with doxorubicin (data not shown).

**V1810 and Genotoxic Drugs Synergistically Decrease Viability of Myeloma Cells**

NF-\( \kappa \)B activation is a mechanism by which myeloma cells escape cell death induced by genotoxic drugs. Having seen that V1810 inhibits NF-\( \kappa \)B activation by melphalan, we examined the effect of combination treatments on the viability of myeloma cells. As shown in Fig. 5B, V1810 and melphalan synergistically induced apoptosis. Incubation of OPM2 cells with 10 \( \mu \)mol/L melphalan or 6 \( \mu \)mol/L V1810 alone only moderately decreased cell viability to 62.6% and 66.7%, respectively. Only the combination treatment with both 10 \( \mu \)mol/L melphalan and 6 \( \mu \)mol/L V1810 was able to decrease cell viability to 11.5% (CI = 0.78 for 10 \( \mu \)mol/L melphalan plus 6 \( \mu \)mol/L V1810; CI = 0.55 for 10 \( \mu \)mol/L melphalan plus 10 \( \mu \)mol/L V1810; Fig. 5B).

**Discussion**

In this study we investigated the antomyeloma effects of V1810, representing a novel class of NF-\( \kappa \)B inhibitors. We show that V1810 potently induces apoptosis both in multiple myeloma cell lines and primary myeloma cells. Moreover, V1810 induces cell cycle arrest by myeloma cells by proteasome-independent inhibition of NF-\( \kappa \)B, and downregulation of Cdk4 and cyclin D1/D2 resulting in hypophosphorylation of retinoblastoma protein. Importantly, V1810 inhibits NF-\( \kappa \)B activation induced by cytotoxic drugs like melphalan and has synergistic antmyeloma activity when combined with melphalan. Pharmacokinetic studies in mice show that concentrations of V1810 effective in vitro can be safely reached in vivo.

We show that V1810 strongly induces apoptosis in multiple myeloma cell lines and primary myeloma cells, but not in PBMCs. PBMCs have low proliferative activity in vitro. Therefore, the specific sensitivity of multiple myeloma cells to V1810 may be in part due to the stronger proliferation of multiple myeloma cell lines. However, primary myeloma cells do not proliferate in vitro and still V1810 strongly induces apoptosis in primary myeloma cells but not in PBMCs. Taken together, V1810 strongly induces apoptosis in multiple myeloma cells. This is in line with previous findings that NF-\( \kappa \)B inhibitors SN50 (6), curcumin (1), and DHMEQ (13, 32) induce apoptosis in multiple myeloma cells. Here we show for the first time that the novel class of NF-\( \kappa \)B inhibitors represented by V1810 potently induces apoptosis in multiple myeloma cells.

We found that V1810-induced apoptosis is associated with inhibition of NF-\( \kappa \)B p65 activity. There are two distinct but interplaying pathways for NF-\( \kappa \)B activation. In the canonical pathway, phosphorylation of I\( \kappa \)B by IKK\( \beta \) leads to I\( \kappa \)B degradation and release of the p50-p65 heterodimer. The alternative pathway is defined by processing of the p52 precursor protein p100, eventually resulting in an active p52-Re\( \kappa \)B heterodimer (2). Both pathways are active in multiple myeloma and it is still to be defined to what extent each of them accounts for multiple myeloma pathophysiology. The frequency of mutations inactivating TRAF3, which is known to inhibit the alternative NF-\( \kappa \)B pathway, point towards a crucial role for the noncanonical pathway in multiple myeloma (2). However, inhibitors of IKK\( \beta \) potently inhibit multiple myeloma cell growth. Considering that IKK\( \beta \) is specific for the canonical pathway, this highlights the importance of the canonical NF-\( \kappa \)B pathway for multiple myeloma cell growth (2, 10, 11). In this study we focused on the effect of V1810 on the canonical pathway quantified by nuclear NF-\( \kappa \)B p65 binding activity. We found that V1810-induced apoptosis is associated with inhibition of NF-\( \kappa \)B p65 activity. Hence, our work further supports the hypothesis that the canonical NF-\( \kappa \)B pathway is of crucial importance to multiple myeloma cell survival and drug resistance.

Recently, Markovina et al. found NF-\( \kappa \)B to be activated by the proteasome-inhibitor resistant pathway in 10 of 14 samples of primary multiple myeloma cells (24). In these specimens, both constitutive NF-\( \kappa \)B activity and NF-\( \kappa \)B activation by bone marrow stromal cells were refractory to bortezomib treatment. This could explain why a substantial percentage of patients are either resistant to bortezomib upfront or become refractory under bortezomib treatment. Here we have shown that V1810 inhibits NF-\( \kappa \)B by a proteasome-independent pathway. V1810 thus meets the need for novel proteasome-independent NF-\( \kappa \)B inhibitors that could overcome drug resistance in patients resistant to bortezomib.

We further found V1810-induced apoptosis to be associated with caspase activation associated with downregulation of Mcl-1, but not cIAP1 and Bcl-2. In contrast, Bcl-2 has been found to be NF-\( \kappa \)B dependent in many models (27–29), and the NF-\( \kappa \)B inhibitor SN50 has been found to decrease levels of both Bcl-2 and cIAP-1, but not Mcl-1 (6). Others, however, have found that inhibition of NF-\( \kappa \)B activity downregulates Bcl-2 in U266 but not in KMS-12PE multiple myeloma cells (13). We concluded that NF-\( \kappa \)B-dependent modulation of antiapoptotic proteins is highly cell line specific and may also vary between NF-\( \kappa \)B inhibitory agents. Further research is needed to elucidate the differential regulation of proapoptotic and antiapoptotic proteins in various multiple myeloma cell lines and subtypes.

In the present study we show that the NF-\( \kappa \)B inhibitor V1810 effectively abrogates expression of Cdk4 and cyclin D1 or D2, but not cyclin D3 in all multiple myeloma cell lines resulting in hypophosphorylation of retinoblastoma protein and cell cycle arrest. As primary multiple myeloma cells arrest in G\( \text{G}_0\)-G\( \text{G}_1\) phase when cultivated ex vivo, cell cycle analysis could only be done in multiple myeloma cell lines. Dysregulation of at least one D-type cyclin (cyclin D1, D2, or D3) is found in virtually all cases of monoclonal gammopathy of undetermined significance and multiple myeloma (33). This had led Bergsagel and Kuehl (34) to propose a new classification of multiple myeloma based...
on genetic translocations and cyclin D expression patterns. Aberrant cyclin D expression occurs as a result of direct immunoglobulin H (IgH) translocations including 11q13 (cyclin D1) and 6p21 (cyclin D3) in about 20% of multiple myeloma cases. In the remaining cases of multiple myeloma, cyclin D is dysregulated indirectly by IgH translocations or mechanisms still to be defined (33, 35, 36). Therefore, it has been hypothesized that cyclin D dysregulation is a key oncogenic event in multiple myeloma formation. Plasma cells with aberrant cyclin D expression are thought to be more susceptible to proliferative signals resulting in a selectively expanding plasma cell population (33, 37). Cyclin D acts by promoting phosphorylation of Rb protein on serine residues Ser780 and Ser807/811 by Cdk4 and 6. This Cdk-specific phosphorylation inactivates Rb, thus releasing E2F and inducing cell cycle progression from G1/S to S phase (38-40). Hideshima and colleagues (33) conclude that the cyclin D/Rb pathway is of crucial importance to multiple myeloma pathogenesis and may be a promising therapeutic target for all molecular subtypes of multiple myeloma. In this study we have shown that V1810 effectively downregulates Cdk4 and cyclin D1/D2, thus leading to decreased phosphorylation of Rb.

It is well established that cyclin D1 is a NF-κB target gene (31). Baldwin (41) summarizes that NF-κB can upregulate expression of cyclin D1, thus promoting Rb hyperphosphorylation and cell cycle progression. We hypothesized that V1810 suppresses cyclin D1/D2 by inhibition of NF-κB. This is consistent with the results of Watanabe et al. (32), who found that the NF-κB inhibitor DHMEQ decreases mRNA levels of cyclin D1 and D2 in multiple myeloma cells. We found that V1810-induced inhibition of baseline NF-κB activity is incomplete. Cyclin D1/D2 expression, in contrast, is completely abrogated after 4 hours of V1810 treatment. Therefore, we cannot exclude the possibility that NF-κB inhibition does not entirely account for V1810-induced cyclin D abrogation. In conclusion, regardless of the extent NF-κB inhibition is responsible for cyclin D downregulation, we have shown that V1810 effectively suppresses NF-κB and the cyclin D/Rb pathway, both of them promising targets for novel multiple myeloma therapies.

We hypothesized that NF-κB–dependent cyclin D inhibition and Rb hypophosphorylation was the mechanism underlying V1810-induced cell cycle arrest, whereas NF-κB–dependent downregulation of antiapoptotic factors such as Mcl-1 accounted for V1810-induced apoptosis. However, Tiedemann et al. (42) have found recently that knockdown of cyclin D1 and D2 by RNA interference not only inhibited cell cycle progression and growth but also induced apoptosis in multiple myeloma cells. Therefore, cyclin D inhibition by V1810 could be a common mechanism accounting for both cell cycle arrest and apoptosis.

We further investigated combination treatments on multiple myeloma cell viability. We did not find any significant additive effects when treating multiple myeloma cells with V1810 and bortezomib (data not shown). Considering that bortezomib has pleiotropic mechanisms of action distinct from NF-κB inhibition (18) and that bortezomib and V1810 inhibit NF-κB by distinct mechanisms, we would expect at least moderate additive effects. However, we did not see significant additive effects. We conclude that bortezomib and V1810 both effectively inhibit NF-κB activity consistent with our hypothesis that NF-κB inhibition was the crucial mechanism of action of both bortezomib and V1810.

We and others have shown previously that genotoxic drugs induce NF-κB in multiple myeloma cells (21, 22). We hypothesized that NF-κB activation was a mechanism by which multiple myeloma cells escape chemotherapy-induced apoptosis and that reversal of NF-κB activation induced by V1810 could result in synergistic effects. We found that V1810 inhibits not only constitutive NF-κB activity but also effectively blocks NF-κB activation induced by melphalan. V1810 entirely reverses NF-κB activation by melphalan. When melphalan and V1810 were combined strong synergistic antitumor effects were observed. We conclude that V1810 overcomes NF-κB–mediated resistance to melphalan by suppressing NF-κB activation. This is consistent with previous findings that bortezomib (21, 23, 43) and inhibitors of protein kinase C (44) sensitize multiple myeloma cells to melphalan by inhibition of NF-κB. Considering that melphalan is still the most important genotoxic drug used in both conventional and high-dose chemotherapy for multiple myeloma, this makes V1810 a promising candidate for the development of novel, effective combination regimens.

In conclusion, in the present study we have shown for the first time that V1810, representing a novel class of proteosome-independent NF-κB inhibitors, induces apoptosis and cell cycle arrest in multiple myeloma cells by specifically targeting the NF-κB/cyclin D/Rb pathway. Importantly, V1810 overcomes drug resistance to melphalan by reversing melphalan-induced NF-κB activation. Pharmacokinetic studies in mice reveal that relevant concentrations of V1810 can be safely reached in vivo. Taken together, V1810 is a promising novel agent for the treatment of multiple myeloma. Further studies will investigate the in vivo efficacy of V1810 alone and particularly in combination regimens with melphalan.

Disclosure of Potential Conflicts of Interest

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