

ORIGINAL ARTICLE

Synergistic antitumor activity of lenalidomide with the BET bromodomain inhibitor CPI203 in bortezomib-resistant mantle cell lymphoma

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Bortezomib therapy has shown promising clinical activity in mantle cell lymphoma (MCL), but the development of resistance to proteasome inhibition may limit its efficacy. To unravel the factors involved in the acquisition of bortezomib resistance *in vivo*, immunodeficient mice were engrafted with a set of MCL cell lines with different levels of sensitivity to the drug, followed by gene expression profiling of the tumors and functional validation of the identified gene signatures. We observed an increased tumorigenicity of bortezomib-resistant MCL cells *in vivo*, which was associated with plasmacytic differentiation features, like interferon regulatory factor 4 (IRF4) and Blimp-1 upregulation. Lenalidomide was particularly active in this subgroup of tumors, targeting IRF4 expression and plasmacytic differentiation program, thus overcoming bortezomib resistance. Moreover, repression of the IRF4 target gene *MYC* in bortezomib-resistant cells by gene knockdown or treatment with CPI203, a BET (bromodomain and extra terminal) bromodomain inhibitor, synergistically induced cell death when combined with lenalidomide. In mice, addition of CPI203 to lenalidomide therapy further decreased tumor burden, involving simultaneous *MYC* and IRF4 downregulation and apoptosis induction. Together, these results suggest that exacerbated IRF4/*MYC* signaling is associated to bortezomib resistance in MCL *in vivo* and warrant clinical evaluation of lenalidomide plus BET inhibitor combination in MCL cases refractory to proteasome inhibition.

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INTRODUCTION

Mantle cell lymphoma (MCL) is an aggressive B-cell neoplasm characterized by the translocation t(11;14)(q13;q32) that leads to aberrant overexpression of cyclin D1 and a number of secondary chromosomal and molecular alterations affecting the cell cycle, senescence and cellular response to DNA damage.¹ Conventional chemotherapy induces high remission rates in previously untreated patients, but relapses within a few years are common, being the median survival 5–7 years.²

The first-in-class proteasome inhibitor bortezomib (VELCADE) is a potent and reversible inhibitor of the 26S proteasome activity, approved in 2006 as second-line treatment for MCL.^{3,4} Bortezomib induces response rates of 30% to 50% in patients with relapsed disease, irrespective of their sensitivity to prior therapy.^{5–7} However, more than half of all MCL patients are intrinsically resistant or develop resistance to bortezomib.² The molecular bases underlying this resistance have not been totally defined in *in vivo* settings, although *in vitro* approaches recently pointed out an important role of oxidative and endoplasmic reticulum stress,⁸ closely linked to the activation of the cytoprotective arm of the unfolded protein response.⁹ In addition, bortezomib-resistant MCL cell lines and primary tumor samples both harbor features characteristic of partial plasmacytic differentiation, including increased expression and transcriptional activity of interferon regulatory factor 4 (IRF4) and of the plasma-cell surface markers CD38 and CD138.¹⁰ Although a physiological role of IRF4 in MCL

pathogenesis requires further elucidation, this crucial regulator of B-cell differentiation and expansion was originally identified as an oncogene transcriptionally activated by t(6;14)(p25;q32) chromosomal translocation in multiple myeloma (MM), where its expression is associated with a poor prognosis.^{11,12} MM cells were further shown to be addicted to an aberrant IRF4 regulatory network, involving an auto-regulatory control mechanism of IRF4 by its target gene *MYC*.¹³

Currently under active investigation for the treatment of B-cell neoplasms, the immunomodulatory drug lenalidomide has recently shown to be well tolerated and to produce rapid and durable responses in heavily pre-treated patients with aggressive, relapsed/refractory MCL, including patients who had failed bortezomib therapy.^{14–17} In addition, a promising overall response rate of lenalidomide in combination therapy has been observed in MCL patients with a poor response to initial treatment.¹⁸ Of interest, the direct antitumor activity of lenalidomide has been shown to rely on the inhibition of the IRF4/*MYC* loop, and to depend on the level of expression of the E3 ligase protein cereblon (CRBN) in both MM cells and in the activated B-cell-like subtype of diffuse large B-cell lymphoma (ABC-DLBCL).^{19–22}

Our aim was to validate IRF4 overexpression and plasmacytic differentiation as markers of bortezomib resistance in *in vivo* xenotransplantation models of MCL, and to assess the possibility of targeting IRF4/*MYC* signaling and overcoming bortezomib resistance using lenalidomide-based therapies.

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MATERIALS AND METHODS

Cell lines and primary samples

The nine human MCL cell lines (Z-138, ZBR, JeKo-1, JBR, REC-1, JVM-2, GRANTA-519, UPN-1 and Mino) used in this study were cultured, routinely tested for *Mycoplasma* infection and checked for their identity as previously described.⁹ Primary MCL cells from four previously untreated patients (see clinical characteristics in Supplementary Table 1), diagnosed according to the World Health Organization (WHO) classification criteria²³ were used. The ethical approvals for this project including the informed consent of the patients were granted following the guidelines of the Hospital Clínic Ethics Committee (Institutional Review Board). Mononuclear cells were isolated from peripheral blood by Ficoll/Hypaque sedimentation (GE Healthcare, Chalfont St Giles, UK) and stored in the Hematopathology Biobank of our institution (Biobanks from CDB-IDIBAPS-Hospital Clínic).

Xenograft mouse model and tumor phenotyping

CB17-severe combined immunodeficiency (SCID) mice (Charles River Laboratories, L'Arbresle, France) were inoculated subcutaneously with 10^7 cells of the indicated MCL cell line, and monitored for tumor growth and vital parameters as previously described.⁹ For lenalidomide (kindly provided by Celgene Corporation, Summit, NJ, USA) and lenalidomide–bortezomib dosing, mice were randomly assigned into cohorts of 3–4 mice each and received by intraperitoneal injection a twice weekly dose of bortezomib (0.15 mg/kg, kindly provided by Millenium/Takeda Oncology, Cambridge, MA, USA), a daily dose of lenalidomide (50 mg/kg), the combination of lenalidomide and bortezomib, or an equal volume of vehicle. In the lenalidomide–CPI203 protocol (CPI203 was kindly provided by Constellation Pharmaceuticals, Cambridge, MA, USA), a total of 22 REC-1 tumor-bearing mice were randomly assigned to cohorts of 5–6 mice, receiving a twice daily intraperitoneal injection of 2.5 mg/kg CPI203, a daily intraperitoneal injection of 50 mg/kg lenalidomide, both agents or an equal volume of vehicle. Between 26 and 29 days post-inoculation, animals were killed according to institutional guidelines and tumor samples were subjected to immunohistochemical staining using primary antibodies against phospho-histone H3 (Epitomics, Burlingame, CA, USA), cleaved caspase-3 (5A1E) and MYC (D84C12) (Cell Signaling Technology, Beverly, MA, USA), IRF4 (M-17) and platelet endothelial cell adhesion molecule-1 (PECAM-1) (M20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD19 (LE-CD19) (Dako, Glostrup, Denmark), Blimp-1 (clone Ros195G/G5, kindly provided by G Roncador, Spanish National Cancer Centre, Madrid, Spain), PAX5 (clone 24, Becton Dickinson, San Jose, CA, USA), CCL3 (R&D Systems, Minneapolis, MN, USA) and CD38 (Sigma-Aldrich, St Louis, MO, USA), as previously described.⁹ Preparations were evaluated using an Olympus DP70 microscope and Cell B Basic Imaging Software (Olympus, Hamburg, Germany).

Gene expression profiling

Total RNA was extracted from representative tumors derived from JeKo-1 ($n = 5$) and JBR ($n = 2$) cell lines using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary RNA was hybridized on the HT HG-U219 GeneChip (Affymetrix, Santa Clara, CA, USA) following standardized protocols. Scanning was processed in a Gene Titan instrument and analyzed with GeneChip Command Console Software (Affymetrix). Raw data were normalized using the Robust Multichip Analysis algorithm implemented in the Expression Console Software v1.1 (Affymetrix). An enrichment pathway analysis was done using the gene set enrichment analysis desktop application version 2.0 (<http://www.broadinstitute.org/gsea/>) in order to find significant gene signatures using experimentally derived custom gene sets (<http://lymphochip.nih.gov/signaturedb/index.html>).²⁴ A two-class analysis with 1000 permutations of gene sets and a weighted metric was used. Gene sets with a false discovery rate below 0.05 were considered to be significant. The leading edge of enriched gene sets, the subset of genes that contributed the most to the enrichment result, were displayed using the Cluster v2.11 and TreeView v1.6 programs (Eisen Laboratory University of California at Berkeley, CA, USA). Primary microarray data are available at the Gene Expression Omnibus of the National Center for Biotechnology Information (GSE51371).

Cytofluorimetric analysis

Phosphatidylserine exposure was quantified by staining with annexin V as described.²⁵ CD38 expression was determined by staining cells with a phycoerythrin-labeled anti-CD38 antibody, using an immunoglobulin G1 isotype-phycoerythrin as negative control (Becton Dickinson). Ten thousand

stained cells per sample were acquired and analyzed in an Attune acoustic focusing cytometer using Attune software (Life Technologies, Carlsbad, CA, USA).

Western blot assays

Five millions cells were lysed in Triton buffer as previously described⁹ and 50 μ g proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA) and probed with antibodies against MYC or IRF4 and the endogenous β -actin (Sigma-Aldrich), followed by incubation with anti-rabbit (Cell Signaling Technology), anti-goat (Santa Cruz Biotechnology) or anti-mouse (Sigma-Aldrich) secondary antibodies. Chemiluminescence detection was done by using ECL system (Thermo Fisher, Waltham, MA, USA) in a mini-LAS4000 device with Image Gauge software (Fujifilm, Tokyo, Japan).

RNA isolation and real-time PCR

Total RNA was extracted using TRIZOL (Life Technologies) following the manufacturer's instructions. One microgram of RNA was retrotranscribed to complementary DNA using moloney murine leukemia virus reverse transcriptase (Life Technologies) and random hexamer primers (Roche, Basel, Switzerland). mRNA expression was analyzed in duplicate by quantitative real-time PCR on the Step one system by using predesigned Assay-on-Demand primers and probes (Life Technologies). The relative expression of each gene was quantified by the comparative cycle threshold method ($\Delta\Delta C_t$). β -Actin was used as an endogenous control.

RNA interference assay

REC-1 cells (7×10^6) were electroporated with a Nucleofector Device (Lonza, Verviers, Belgium, program A-032) Ingenio Electroporation Solution (Mirus, Madison, WI, USA) containing either 2.5 μ M of a Silencer Select Predesigned small interfering RNA (siRNA) targeting the MYC gene, a mix of two Silencer Select Predesigned siRNA targeting the IRF4 gene or a non-silencing (scramble) siRNA (Ambion, Austin, TX, USA), or 200 nM of a mix of three different predesigned siRNA-targeting CRBN gene or a control duplex siRNA (Integrated DNA Technologies, Leuven, Belgium). After transfection, cells were transferred to culture plates for 6 h before experiments were set up.

Cell proliferation assay

MCL primary cells (1.5×10^5) and cell lines (4×10^4) were incubated as indicated with lenalidomide and/or CPI203. MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich) was added for 2–6 additional hours before spectrophotometric measurement. Each measurement was made in triplicate. Values were represented using untreated control cells as reference. The GI_{50} was calculated as the concentration that produced 50% growth inhibition. Combination indexes (CIs) were calculated by using the Calcsyn software version 2.0 (Biosoft, Ferguson, MO, USA). The interaction between two drugs was considered synergistic when $CI < 1$.

Statistical analysis

Data are represented as mean (\pm s.d. or s.e.m) of three independent experiments. All statistical analyses were done using GraphPad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between two groups of samples were evaluated by the Student's *t*-test or nonparametric Mann–Whitney test. Results were considered statistically significant when $P < 0.05$.

RESULTS

Bortezomib resistance correlates with plasmacytic differentiation and increased tumorigenicity *in vivo*

To investigate the impact of bortezomib resistance on MCL cell tumorigenicity, three MCL cell lines with acquired or constitutive resistance to bortezomib (JBR, ZBR and REC-1), as well as four bortezomib-sensitive cell lines (JeKo-1, Z-138, JVM-2 and GRANTA-519) were inoculated in immunodeficient mice to generate the corresponding MCL xenograft models. The generation and characterization of JBR and ZBR, derived from the parental, bortezomib-sensitive cell lines JeKo-1 and Z-138, respectively, have been described previously.⁹ Although these cell lines did not

show noticeable differences in their growth properties *in vitro* (data not shown), a significant increase in tumor burden was observed 4 weeks after the inoculation in animals engrafted with the bortezomib-resistant cell lines compared with the mice bearing bortezomib-sensitive tumors (Figure 1a). To investigate

the molecular basis of this phenomenon, we compared the gene expression profiling of tumors derived from the representative bortezomib-sensitive cell line JeKo-1 and its derived bortezomib-resistant subclone JBR. A first inspection of gene expression profiling results indicated the upregulation of genes related to

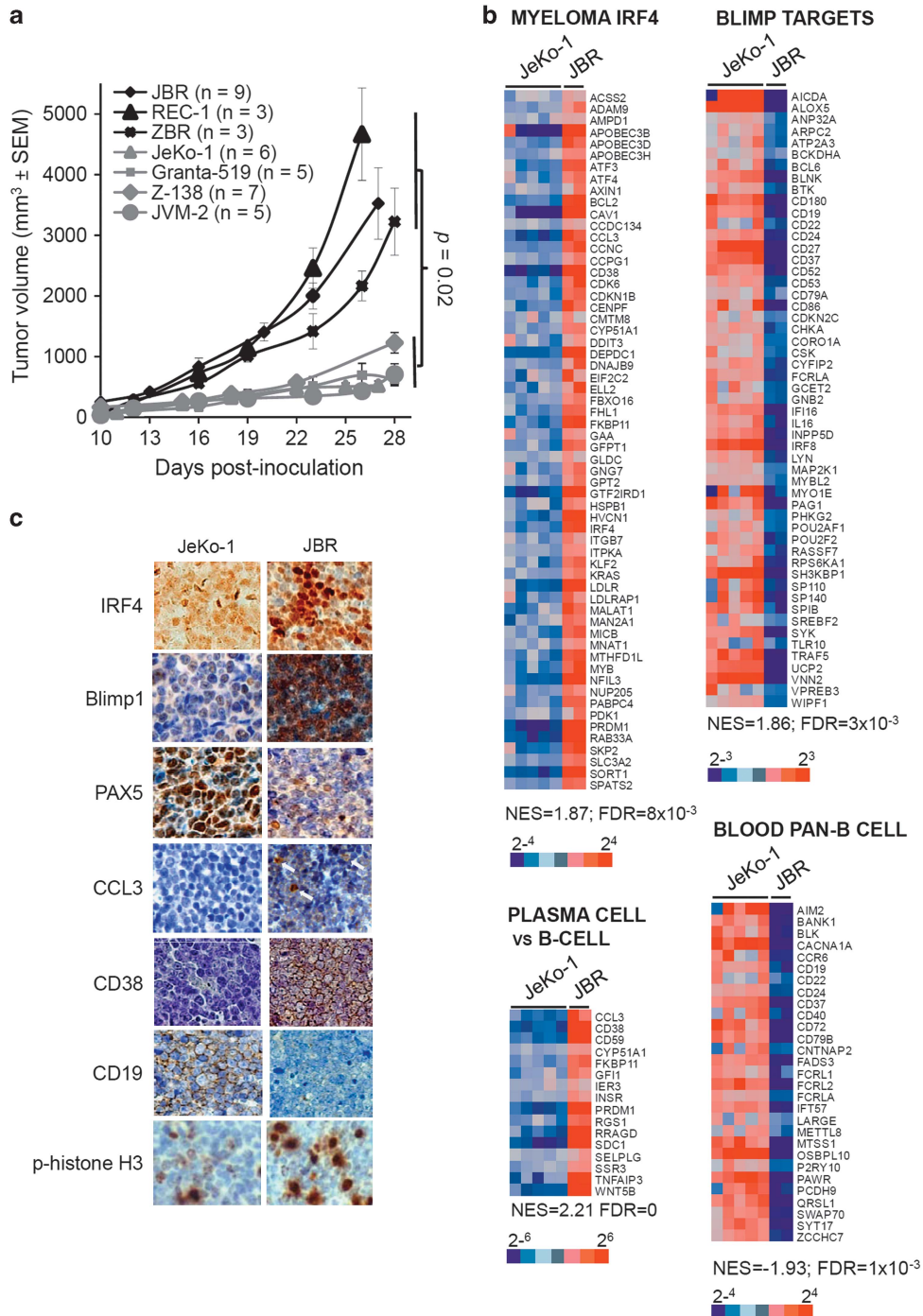


Figure 1. Increased tumorigenicity of bortezomib-resistant cell lines is associated with plasmacytic differentiation. (a) A set of bortezomib-resistant (black curves) and bortezomib-sensitive (gray curves) MCL cell lines were subcutaneously inoculated into 3–9 SCID mice and tumor growth was recorded for up to 27 days. (b) Gene expression signatures of plasma-cell differentiation distinguish tumors derived from the representative, bortezomib-resistant cell line JBR, from tumors derived from the parental, bortezomib-sensitive cell line JeKo-1. The leading edge of each gene set is displayed in a heat map. NES, normalized enrichment score. (c) Changes in the expression of selected plasma-cell markers were confirmed by immunohistochemical staining of consecutive sections from tumors representative of each subgroup. Bortezomib-resistant tumors harbored several hallmarks of plasmacytic differentiation including increased IRF4 and Blimp-1 levels and decreased of PAX5 expression, accompanied by an increase in mitotic index revealed by p-histone H3 staining and by the apparition of CCL3-secreting cells (white arrows) (magnification × 80).

plasma-cell differentiation and the downregulation of B-cell genes in JBR-derived tumors. To determine the significance of these changes, we performed gene set enrichment analysis with well-defined gene signatures of plasma-cell differentiation, as previously described.¹⁰ All of these signatures were significantly

enriched in the tumors derived from JBR cells, indicating a shift from a B-cell to a plasmacytic gene expression program (Figure 1b). Immunohistological analysis confirmed that an increased mitotic index, as assessed by phospho-histone H3 labeling, was associated with marked upregulation of the

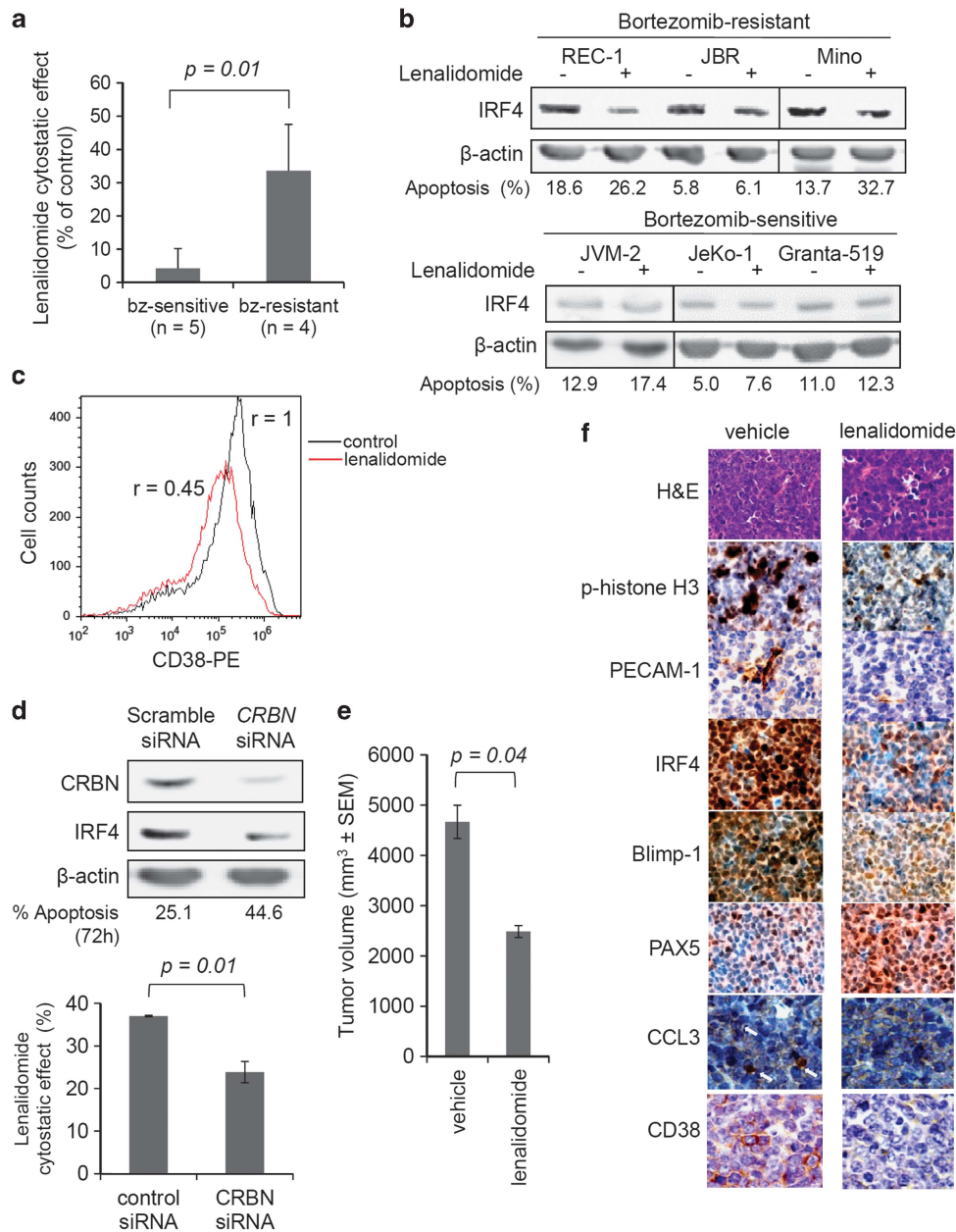


Figure 2. Antitumoral activity of lenalidomide is mediated by inhibition of the plasmacytic differentiation program in bortezomib-resistant MCL. **(a)** A set of nine MCL cell lines were incubated for 72 h with $1 \mu\text{M}$ lenalidomide and cytotoxicity was assessed by MTT assay. The relative number of proliferating cells compared with untreated cells is presented as the mean (s.d.) of triplicate assays. **(b)** IRF4 protein downregulation after lenalidomide treatment ($1 \mu\text{M}$, 72 h) was analyzed by western blot in six representative bortezomib-resistant and bortezomib-sensitive MCL cell lines. Cell viability was assessed by cytofluorimetric detection of Annexin V-positive cells. **(c)** REC-1 cells treated as in **b** were analyzed by flow cytometry for their membrane levels of CD38; r values refer to the mean fluorescence ratio between the mean fluorescence intensity of the CD38-phycoerythrin (PE) signal in treated sample and untreated sample and an isotypic control, using untreated cells as a calibrator. **(d)** REC-1 cells were electroporated in the presence of CRBN or negative control siRNA and CRBN and IRF4 protein levels were evaluated by western blot. β -Actin was used as a loading control (upper panel). Transfected cells were either untreated or treated with $5 \mu\text{M}$ lenalidomide for 72 h and cell proliferation was evaluated by MTT assay (lower panel). **(e)** REC-1 cells (10^7 cells per mouse) were subcutaneously inoculated into the right flank of SCID mice. Tumor-bearing mice ($n = 3-4$ mice per group) received intraperitoneal injections of 50 mg/kg lenalidomide or an equal volume of vehicle, 5 days a week, for 3 weeks. Tumor volumes were measured at the end of the treatment period with external calipers. **(f)** Representative tumor samples were then assessed by immunohistochemical staining for mitotic index (p-histone H3), angiogenesis (platelet endothelial cell adhesion molecule-1 (PECAM-1)), plasmacytic differentiation (IRF4, CD38, Blimp-1 and CCL3) and B-cell related markers (PAX-5). White arrows: CCL3-producing cells (magnification $\times 800$).

plasmacytic markers IRF4, Blimp-1 and CD38, together with a loss of the common B-cell markers PAX5 and CD19, in JBR versus JeKo-1 tumors (Figure 1c and Supplementary Figure S1A). IRF4 staining, scored by an expert pathologist in three degrees referring to the level of expression observed in plasma cells, revealed that in JeKo-1 tumors, 5% and 60% of the cells were scored as 3+/3 and 1+/3+, respectively, contrasting with JBR tumors where 50% of the cells were scored as 3+/3 and 40% of the cells as 2+/3+ (Figure 1c). In accordance with previously published data,¹⁰ IRF4 was found to be a crucial regulator of MCL sensitivity to bortezomib as its siRNA-mediated downregulation significantly increased REC-1 cell response to the proteasome inhibitor (Supplementary Figure S2A). Further, as part of the IRF4 gene signature, JBR, but not JeKo-1 tumors exhibited an eightfold increase in CCL3 mRNA and CCL3 protein expression together with a sevenfold increase in *IRF4* mRNA (Figure 1c, Supplementary Figures S1A, S2B and S2C). In agreement with a physiological role of this chemokine in the regulation of IRF4/MYC signaling and MCL resistance to bortezomib, treatment of JeKo-1 cells with recombinant CCL3 was shown to increase MYC expression and to limit the pro-apoptotic activity of bortezomib in these cells (Supplementary Figures S2D and S2E). Together, these results suggest that the acquisition of a plasmacytic phenotype by bortezomib-resistant MCL cells may confer increased tumorigenicity *in vivo*.

Lenalidomide counteracts IRF4 upregulation and plasmacytic differentiation program in bortezomib-resistant cell lines and tumors

As lenalidomide activity has been shown recently to rely on IRF4 downregulation in MM and ABC-DLBCL,^{19,21} we assessed whether the drug could exert such activity in MCL cells resistant to bortezomib. Figure 2a and Table 1 show an inverse correlation between the response to lenalidomide and the degree of sensitivity to bortezomib in a panel of nine MCL cell lines. Indeed, lenalidomide was found to be significantly ($P=0.01$) more active in bortezomib-resistant cells where it exerted an average cytostatic effect of 33.6%, when compared with the bortezomib-sensitive subset of cell lines (mean effect: 4.8%). This phenomenon was associated with a remarkable inhibition of IRF4 overexpression in bortezomib-resistant cells exposed to lenalidomide, while the lower, basal IRF4 levels observed in the bortezomib-sensitive cells were almost unmodified by the treatment (Figure 2b). Accordingly, the cytofluorimetric quantification of CD38 revealed a 55% decrease in the expression of this marker in REC-1 cells exposed to lenalidomide, when compared with untreated cells (Figure 2c). To explore the requirement of CRBN for lenalidomide-mediated cytotoxicity and IRF4 downregulation, we selectively knocked down the *CRBN* gene in REC-1 cells by a siRNA approach prior lenalidomide treatment. When compared with cells transfected with a non-targeting siRNA, the combination of three different oligonucleotides against *CRBN* effectively blocked the synthesis of the protein, leading to IRF4 downregulation and a decrease in the antitumor activity of lenalidomide from 37 to 23.9% ($P=0.01$; Figure 2d). Accordingly, in the absence of CRBN, lenalidomide-mediated decrease in IRF4 protein levels was partially impaired after a 72-h exposure to the drug (Supplementary Figure S3). Of note, IRF4 downregulation in CRBN knockdown cells was also associated with a slight increase (+19.5%) in the rate of apoptotic cells, underlying the dependence of REC-1 toward this factor for their survival (Figure 2d). Thus, as previously described in MM and ABC-DLBCL cell lines, the *in vitro* activity of lenalidomide in bortezomib-resistant MCL cell lines may involve the downregulation of IRF4 in a CRBN-dependent manner.

To validate these results *in vivo*, CB17-SCID mice bearing REC-1 cell-derived tumors were randomly assigned to treatment with

Table 1. Drug sensitivity of MCL cell lines

Cell line	Bortezomib sensitivity	Lenalidomide cytotoxicity at 72 h (% of control)	CPI203 GI_{50} at 72 h ($\mu\text{M} \pm \text{s.d.}$)
JVM-2	Sensitive	< 1	0.21 \pm 0.02
Z-138	Sensitive	8.5	0.14 \pm 0.01
Granta-519	Sensitive	< 1	0.71 \pm 0.03
JeKo-1	Sensitive	< 1	0.26 \pm 0.05
UPN-1	Sensitive	12.6	0.08 \pm 0.002
REC-1	Resistant	45.7	0.21 \pm 0.03
Mino	Resistant	16.4	0.06 \pm 0.03
JBR	Resistant	44.1	0.18 \pm 0.05
ZBR	Resistant	28.1	0.19 \pm 0.06

Abbreviation: MCL, mantle cell lymphoma.

lenalidomide (50 mg/kg, daily) or vehicle. As shown on figure 2e, after 3 weeks of treatment, tumors from lenalidomide-treated mice had a significant ($P=0.04$) lower volume than tumors from the vehicle group. The immunohistochemistry analysis of representative tumor sections revealed that lenalidomide therapy efficiently reduced the hallmarks of bortezomib resistance and aggressiveness in REC-1 tumors, such as phospho-histone H3, IRF4, CD38, Blimp-1 and CCL3, and restored the expression of PAX5 (Figure 2f and Supplementary Figure S1B). Western blot analysis of representative whole tumor specimens, indicated that IRF4 and its target gene MYC experimented a 35% and a 85% protein decrease after lenalidomide therapy, respectively (Supplementary Figure S1E). In addition, the interferon-regulated genes *CXCL10* and *CXCL11* were the most downregulated genes in lenalidomide-receiving tumors among a panel of 90 genes related to immune regulation (data not shown), in agreement with previous findings in ABC-DLBCL cells.²⁰ Finally, lenalidomide was also able to reduce the length and number of intramural tumor blood vessels, as shown by a reduction in platelet endothelial cell adhesion molecule-1 staining (Figure 2f and Supplementary Figure S1B). Thus, lenalidomide activity appeared to be stronger in MCL cell lines and tumors resistant to bortezomib, mediated by the downregulation of IRF4 and plasma-cell-related antigens and cytokines, and impaired angiogenesis.

Lenalidomide is able to overcome bortezomib resistance *in vivo*

In an attempt to assess whether lenalidomide could reverse MCL resistance to bortezomib *in vivo*, mice inoculated with REC-1 cells as above were assigned to four different treatment arms: bortezomib 0.15 mg/kg twice per week, lenalidomide 50 mg/kg daily, a combination of both agents or equal volume of vehicle. Although administration of bortezomib as a single agent did not affect tumor burden, mice receiving lenalidomide showed a 45% reduction in tumor growth when compared with vehicle group (Figure 3a). This effect was greatly enhanced by the addition of bortezomib to lenalidomide, reaching a 37% and 67% tumor decrease when compared with lenalidomide and vehicle arms, respectively, in the absence of drug-related toxicity. Accordingly, although tumor metabolism was almost unaffected by bortezomib treatment, lenalidomide and lenalidomide plus bortezomib therapies led to a 43% and a 94% reduction in glucose uptake, respectively, indicative of synergy between these two compounds *in vivo* (Figure 3b). The histological analysis of the corresponding tumors confirmed a stronger reduction of tumor mitotic index by the combination therapy, together with a profound downregulation of IRF4 (Figure 3c and Supplementary Figure S1C). Importantly, although some degree of apoptosis could be detected by means of activated-caspase-3 staining in the lenalidomide, but not in the control or bortezomib arms, the tumors from combination-receiving mice showed a remarkable accumulation of apoptotic

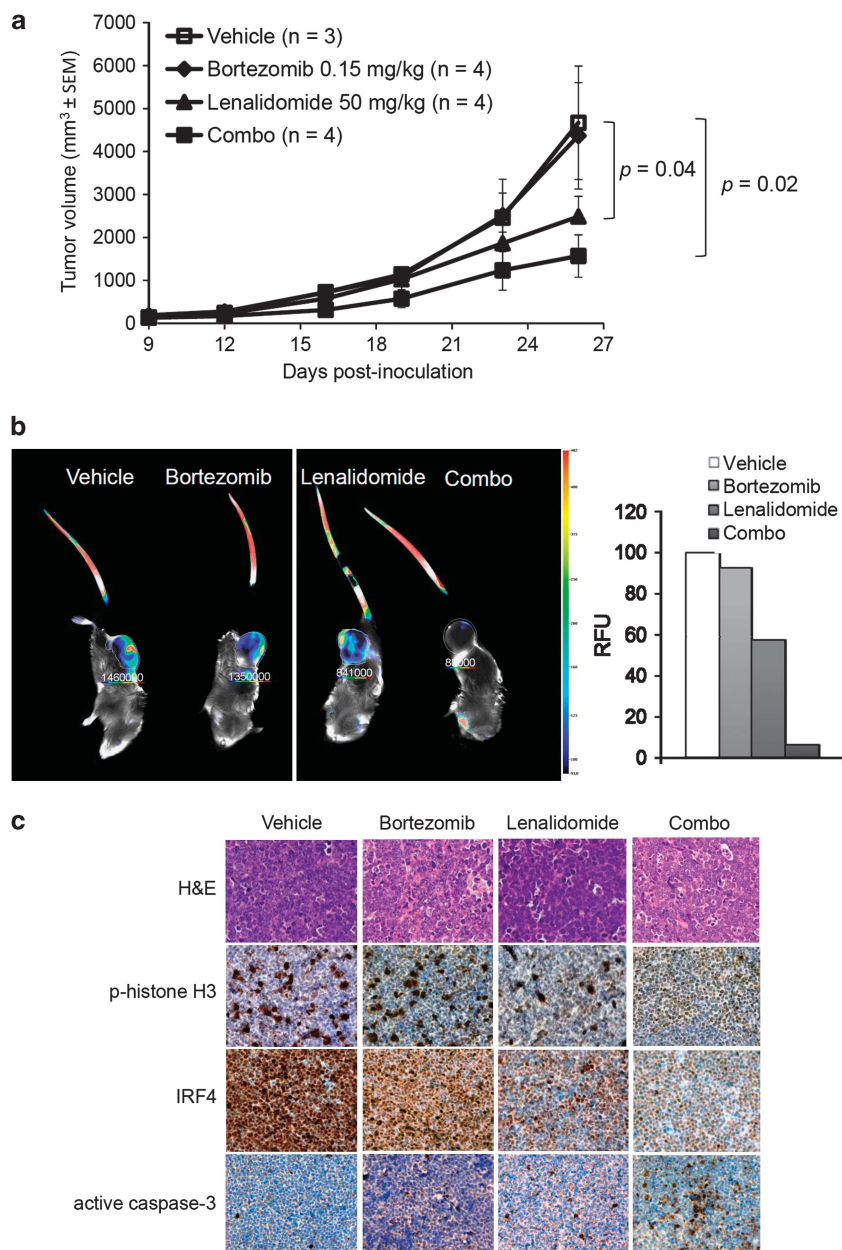


Figure 3. Lenalidomide overcomes bortezomib resistance *in vivo*. **(a)** Mice were inoculated with REC-1 cells as previously described and began treatment at day 9 post-inoculation with 50 mg/kg lenalidomide, 0.15 mg/kg bortezomib, both agents or equal volume of vehicle. Lenalidomide was administered 5 days a week and bortezomib twice a week, for up to 18 days. **(b)** Left panel, intratumoral glucose uptake was evaluated in representative mice injected intravenously with an IR800-labeled 2-deoxy glucose probe 24 h prior killing, and visualized with an Odyssey infra-red scanner (Li-Cor, Lincoln, NE, USA). Right panel, relative fluorescence quantification by means of the Image Studio software (Li-Cor) shows markedly reduced glucose uptake in tumor masses from mice receiving lenalidomide or the lenalidomide–bortezomib combination, when compared with either vehicle- or bortezomib-treated animals. **(c)** Immunohistochemical staining of consecutive sections from representative tumors, pointing out the synergistic downregulation of IRF4 and proliferation, and induction of apoptosis by the lenalidomide and bortezomib combination in REC-1-derived tumors (magnification $\times 200$).

cells (Figure 3c and Supplementary Figure S1C). Taking together, these results suggest that the bortezomib–lenalidomide combination is able to overcome bortezomib resistance of MCL tumors *in vivo*, via the inhibition of IRF4 expression, impaired cellular proliferation and induction of apoptosis.

A role for MYC in the response of bortezomib-resistant cells to lenalidomide

According to the above results, bortezomib-resistant MCL may present several similarities with myeloma cells in terms of IRF4

expression and response to lenalidomide. As MYC has been described as a direct target gene of IRF4 in this latest model,¹³ we further assessed the levels of this oncogene in bortezomib-resistant MCL tumors and its role in lenalidomide-treated cells. Figures 4a and b show that MYC was upregulated in JBR versus JeKo-1 tumors, in agreement with the difference in mitotic index previously observed between the two sets of tumors. Accordingly, a two- to threefold increase in MYC mRNA levels was detected by quantitative reverse transcriptase-PCR in JBR and REC-1-derived tumors when compared with JeKo-1-derived specimen, indicative of a transcriptional upregulation of the gene (Supplementary Figure S4). To further

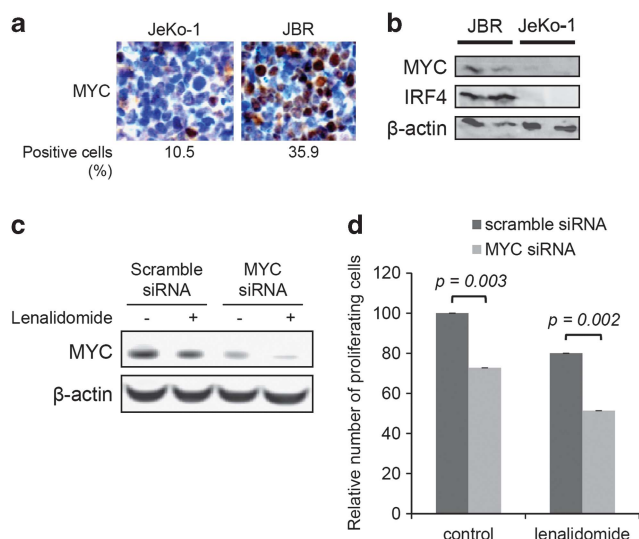


Figure 4. Role of MYC expression in MCL cell sensitivity to bortezomib and lenalidomide. **(a)** Immunohistochemical detection of increased MYC nuclear levels in bortezomib-resistant vs bortezomib-sensitive tumors (magnification $\times 800$). **(b)** Western blot analysis of JBR and JeKo-1 tumor samples (two samples per group) confirming the accumulation of MYC, in addition to IRF4, in bortezomib-resistant MCL tumors. **(c)** MYC siRNA and scrambled non-silencing siRNA were transferred to REC-1 cells by electroporation and MYC protein levels were evaluated by western blot. β -Actin was used as a loading control. **(d)** Transfected cells were either untreated or treated for 48 h with $1 \mu\text{M}$ lenalidomide, after which time cell proliferation was evaluated by MTT assay.

characterize the role of MYC in the response of bortezomib-resistant MCL cells to lenalidomide, we used a siRNA approach to selectively downregulate MYC protein synthesis in REC-1 cells, followed by a 48-h treatment with lenalidomide. The specific MYC-targeting oligonucleotide used was able to abrogate MYC expression in REC-1 cells, compared with cells transfected with a non-targeting siRNA (Figure 4c). MYC knockdown was then shown to induce a 20% reduction in REC-1 cell proliferation. When combined with lenalidomide, we observed a complementary downregulation of MYC and an enhanced antiproliferative effect that reached 50% (Figures 4c and d). Thus, in addition to IRF4 expression, MYC upregulation appears to be a hallmark of MCL resistance to bortezomib, and may condition MCL response to lenalidomide.

The BET bromodomain inhibitor CPI203 exerts cytostatic effect and synergistically increased lenalidomide-induced apoptosis in MCL cells

Based on the recent demonstration that pharmacologic inhibition of MYC is achievable through targeting bromodomain and extra terminal (BET) family of chromatin adapters,^{26,27} we assessed the possible role of MYC in the response of MCL to lenalidomide by evaluating the anti-proliferative activity of CPI203, a potent, selective and competitive small molecule inhibitor of BET proteins.^{28,29} As shown in Figure 5a and Table 1, CPI203 exerted a cytostatic effect in the nanomolar range in all the nine MCL cell lines analyzed, independently of their sensitivity to either bortezomib or lenalidomide, with a mean GI_{50} value at 72 h of $0.23 \mu\text{M}$ (range: $0.06\text{--}0.71 \mu\text{M}$). At this concentration, the cytotoxicity of the compound in normal peripheral blood mononuclear cells from healthy donors was below 25%, thus demonstrating its selectivity. Treatment with $0.5 \mu\text{M}$ CPI203 in two representative bortezomib-resistant MCL cell lines with high MYC basal levels (REC-1, JBR) and two representative bortezomib-sensitive MCL cell lines harboring lower expression of the oncogene (GRANTA-519 and JVM-2), effectively reduced MYC expression in all the samples

without inducing apoptosis (Figure 5b). This effect was mediated by the repression of MYC gene transcription, as confirmed by quantitative reverse transcriptase-PCR (data not shown). We then assessed whether CPI203 treatment could enhance MCL response to lenalidomide. The four bortezomib-resistant MCL cell lines REC-1, MINO, JBR and ZBR, together with four lenalidomide-sensitive primary cultures, were treated with a 0.1 or $0.5 \mu\text{M}$ dose of CPI203, followed by a 72-h exposure to 1 or $5 \mu\text{M}$ lenalidomide, and the CI values were calculated. At both 0.1 and $0.5 \mu\text{M}$ doses, CPI203 exerted a synergistic cytotoxic effect with lenalidomide $5 \mu\text{M}$ on all the samples analyzed. For lenalidomide–CPI203 $0.1 \mu\text{M}$ combination, the median CI was 0.35 (range: $0.19\text{--}0.57$) in the cell lines and 0.39 (range: $0.07\text{--}0.56$) in the primary cultures. For lenalidomide–CPI203 $0.5 \mu\text{M}$ combination, the median CI was 0.5 (range: $0.29\text{--}0.55$) in the cell lines and 0.52 (range: $0.05\text{--}0.64$) in the primary cultures. Figure 5c shows representative data obtained from REC-1 cells and primary cells from MCL patient #4. Consistently, while lenalidomide alone could partially reduce MYC expression, together with IRF4, in REC-1 cells, the combination of both agents almost completely abrogated the expression of both factors (Figure 5d). Of note, CPI203 single-agent treatment also appeared to slightly affect IRF4 expression (Figure 5d), underlying a possible retro-control of IRF4 expression by MYC in bortezomib-resistant MCL cells, as previously reported in myeloma cells.³⁰ Importantly, lenalidomide or CPI203 alone activated the apoptotic program in these cells only marginally (see Figures 2b and 5b), whereas the combination of both drugs achieved an apoptotic response in $>70\%$ of the cells (Figure 5d), suggesting that simultaneous targeting of IRF4 and MYC by lenalidomide and BET inhibition efficiently activates the cell death program in MCL cells resistant to bortezomib.

The CPI203–lenalidomide combination synergistically inhibits the growth of bortezomib-resistant tumors *in vivo*

To further characterize *in vivo* the synergistic role of lenalidomide and CPI203, SCID mice inoculated with REC-1 cells were randomly assigned into drug-treated (lenalidomide 50 mg/kg daily, CPI203 2.5 mg/kg BID, combo) and vehicle-treated groups. As shown in Figure 6a, while lenalidomide alone achieved a 41% reduction in tumor volume when compared with vehicle group, this effect reached 44% and 62% in mice receiving CPI203 and the lenalidomide–CPI203 combination, respectively. Similar results were obtained using a JBR-induced xenotransplant model of MCL (data not shown). Accordingly, tumor glucose uptake was reduced to 40–45% in animals treated with either lenalidomide or CPI203, while this reduction reached 86% in the combo group (Figure 6b). Immunohistochemical analysis of the corresponding tumors confirmed a synergistic decrease in the mitotic index, together with the almost complete disappearance of both MYC- and IRF4-positive cells, and an accumulation of cleaved caspase-3-positive cells in the combo-receiving group (Figure 6c and Supplementary Figure S1D). These results confirmed our *in vitro* data, showing that the combination of lenalidomide with the BET inhibitor CPI203 synergistically augments the antitumoral properties of each single agent, thanks to the abrogation of MYC and IRF4 expression and the induction of apoptosis.

Altogether, our results suggest that exacerbated IRF4/MYC signaling is a hallmark of bortezomib resistance in *in vivo* models of MCL and that dual inhibition of IRF4 and MYC by means of lenalidomide and BET bromodomain inhibitor is a promising strategy that may warrant clinical activity in MCL cases refractory to proteasome inhibition.

DISCUSSION

The mechanism of action of bortezomib in MCL is complex, as it affects many cellular processes as diverse as BCL-2 protein

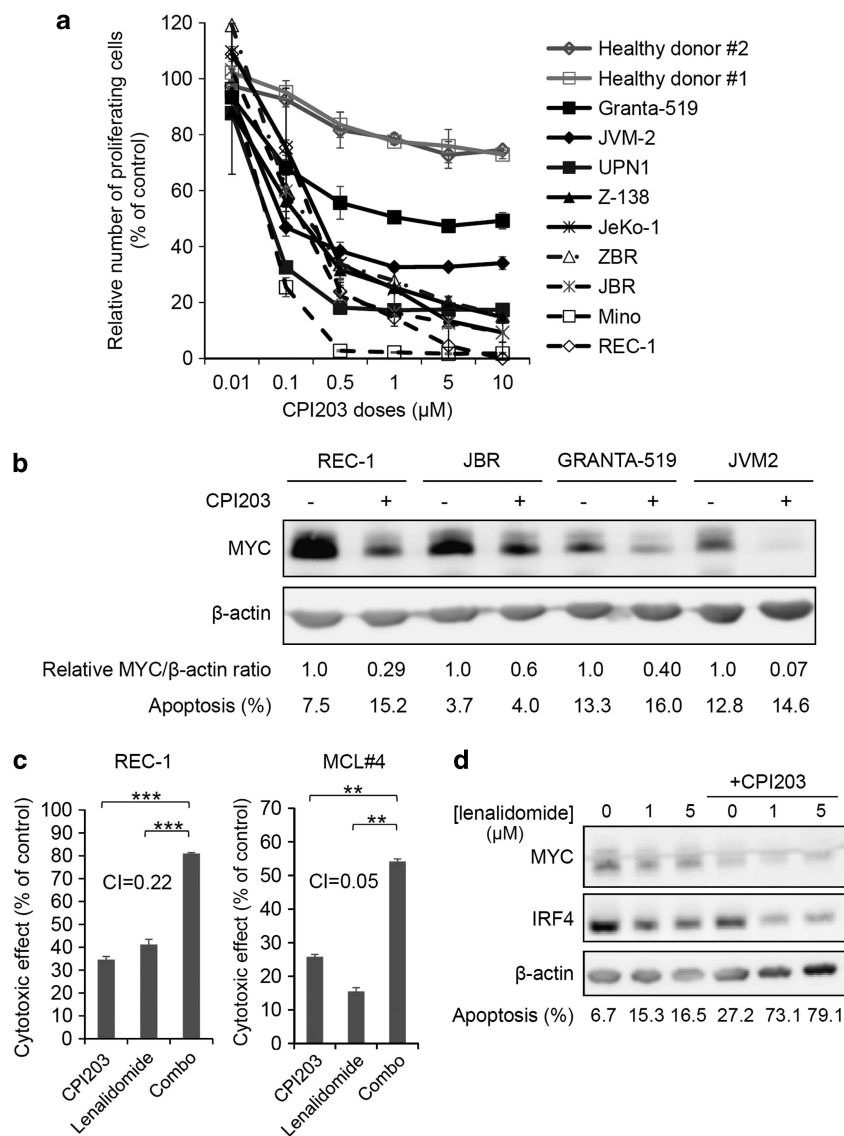


Figure 5. CPI203 synergistically induces apoptosis with lenalidomide in bortezomib-resistant cells. **(a)** A set of nine MCL cell lines and two peripheral blood mononuclear cell (PBMC) cultures from healthy donors were incubated for 72 h with increasing concentrations of CPI203 and cytotoxicity was assessed by MTT assay. The relative number of proliferating cells compared with control (untreated cells) are presented as the mean (s.d.) of triplicate assays. **(b)** Decreases in MYC protein levels after CPI203 (500 nM, 24 h) treatment was analyzed by western blot in four MCL cell lines. Cell viability was assessed by cytofluorimetric detection of Annexin V-positive cells. **(c)** Synergistic effect of lenalidomide and CPI203 combination in MCL cell lines and primary samples. Cells were treated for 72 h with 0.1–0.5 µM CPI203 and/or 1–5 µM lenalidomide. Cytotoxicity was determined by the MTT assay, and the CI values were calculated. Shown are the cytotoxicity and the CI values calculated after treating REC-1 cell with 0.1 µM CPI203 and 5 µM lenalidomide and primary cells from MCL case #4 with 0.5 µM CPI203 and 5 µM lenalidomide. **(d)** REC-1 cells were treated as above with 0.5 µM CPI203 and 1–5 µM lenalidomide. MYC and IRF4 levels were determined by western blot, and cell viability was determined by flow cytometry detection of annexin V binding.

modulation, reactive oxygen species production, endoplasmic reticulum stress and unfolded protein response signaling.⁴ This diversity of effects may explain, at least in part, the gap between the promising activity of the drug reported in the first preclinical studies and its clinical efficacy. This also illustrates the need for *in vivo* models of bortezomib resistance to validate the markers and combinational therapies identified by *in vitro* approaches.

Here, by analyzing the *in vivo* properties of a panel of seven MCL cell lines, including previously described cell lines with spontaneous or acquired resistance to the proteasome inhibitor,⁹ we describe for the first time that drug-resistant MCL cell lines have increased tumorigenic properties *in vivo*, and that the increased aggressiveness of these tumors correlates with the expression of a plasmacytic differentiation program, being IRF4

a major regulator of this phenomenon.^{31,32} This observation not only confirms the recent identification of plasma-cell differentiation as a surrogate of bortezomib resistance in MCL,¹⁰ but it also links for the first time this phenotype with increased tumor angiogenesis, mitotic indexes and MYC expression levels.

Indeed, although MYC is not included in the different gene sets identified either by our *in vivo* gene set enrichment analysis or by the previous *in vitro* study,¹⁰ we detected a threefold increase in MYC mRNA levels and a remarkable nuclear accumulation of the protein in JBR tumors. The evidence presented here suggests that the major mechanism of MYC upregulation in bortezomib-resistant tumors is the overexpression IRF4, as the downregulation of the oncogene is systematically observed after specific IRF4 knockdown *in vitro* (data not shown) and following the

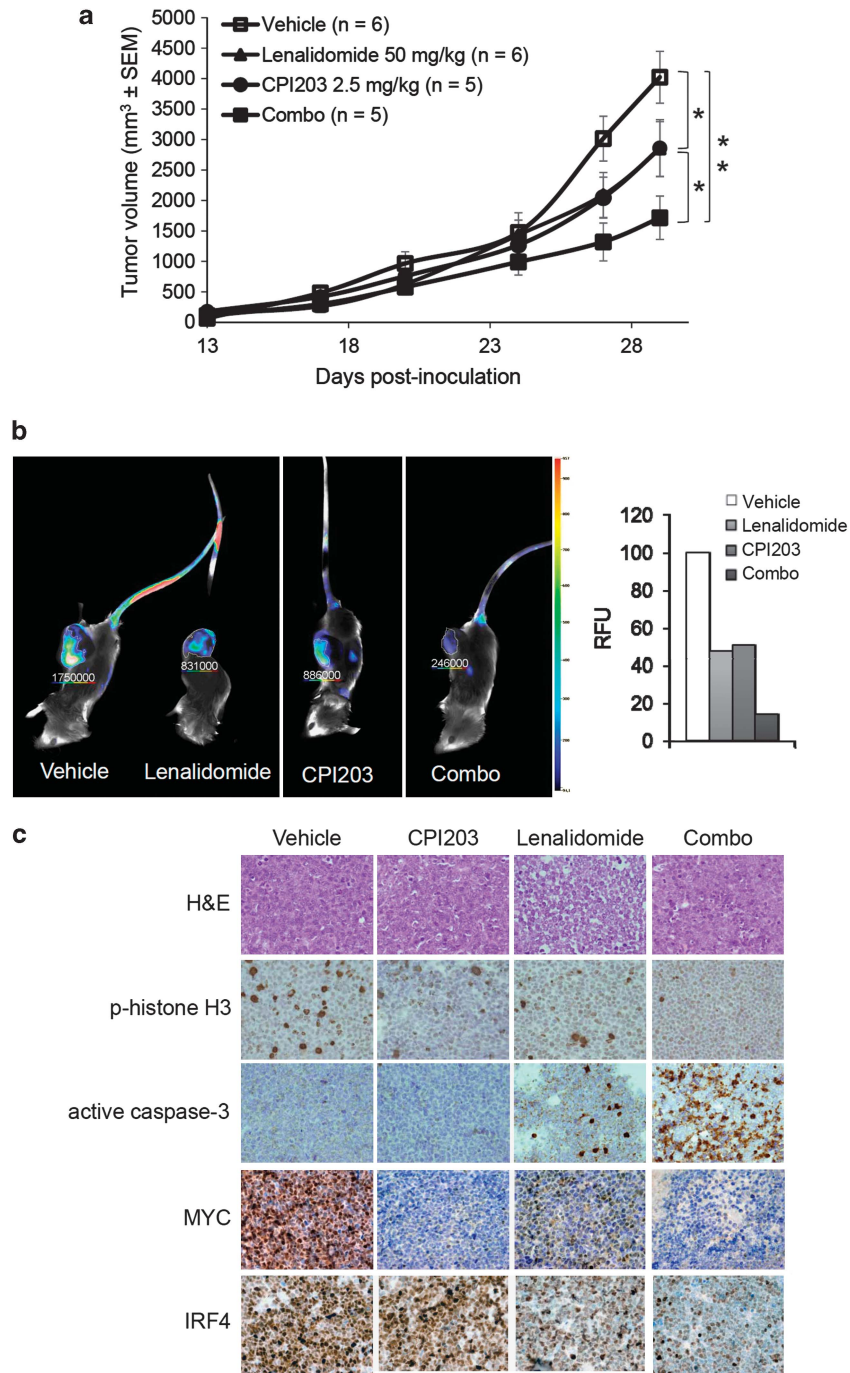


Figure 6. CPI203 plus lenalidomide synergistically inhibits the growth of bortezomib-resistant tumors. **(a)** Twenty-four SCID mice were inoculated with 10^7 REC-1 cells as previously described. Tumor-bearing mice were randomly assigned to one of the following treatment arms ($n = 5-6$ mice per group): lenalidomide 50 mg/kg daily, CP203 2.5 mg/kg BID, both agents or equal volume of vehicle, for 16 days. Tumor volumes were recorded each 3-4 days as previously. **(b)** Odyssey software pictures and corresponding fluorescence quantifications from representative mice at the day of killing. **(c)** Immunohistochemical labeling of p-histone H3, activated caspase-3, MYC and IRF4 in consecutive tissue sections from four representative tumor specimens (magnification $\times 200$), illustrating the synergistic downregulation of IRF4, MYC and proliferation, and induction of apoptosis in tumors from mice receiving lenalidomide-CPI203 combination.

administration of lenalidomide *in vivo*. Beside the downregulation of MYC, this activity of lenalidomide toward IRF4 expression may allow MCL tumors to partially reverse the plasmacytic differentiation and to recover their sensitivity to bortezomib cytotoxic effect. Although such a decrease in IRF4 levels has been shown to be sufficient to induce apoptosis in MM and ABC-DLBCL cell lines exposed to similar doses of the immunomodulatory drug,^{13,19,20} our present results show that lenalidomide alone

exerts mostly cytostatic effects in MCL cultures. In turn, we found that dual extinction of both IRF4 and MYC is required to achieve apoptotic processing *in vitro* and *in vivo*, and that both factors may represent important targets for the therapy of MCL patients, as described for MM patients.³³ Arguing in favor of this notion, IRF4 has been shown to be required for the survival of bortezomib-resistant MCL cells,¹⁰ and overactivation of IRF4/MYC axis has been proposed as the 'Achilles' heel' of MM and ABC-DLBCL cells.^{13,20}

MYC overexpression is associated with shorter survival in MCL patients, especially in those rare double-hit cases with a MYC-activating translocation and pleomorphic blastoid morphology.³⁴ First attempts of specific pharmacological inhibition of MYC activity were focused on the inhibition of its interaction with its co-factor MAX complexes by a small molecule approach that, despite a remarkable efficiency *in vitro*, could not maintain a significant inhibitory activity in *in vivo* models of Burkitt lymphoma with t(8;14)(q24;q32) activating translocation.³⁵ More recently, the use of (+)-JQ1, a highly potent, selective and cell permeable inhibitor of BRD4, a member of the BET family of chromatin adaptors, has shown antitumor activity in MM xenograft models, mediated by transcriptional inhibition of MYC.^{26,36} BET proteins are transcriptional regulators that selectively recognize and bind to acetylated lysine residues in histones, and have an important role in cellular maintenance, proliferation, differentiation and activation. Their deregulation may result in various cancers, inflammatory diseases, metabolic diseases and tissue degeneration, involving the abnormal stimulation of transcriptional elongation of certain target genes including MYC and BCL2 oncogenes.³⁶ Further studies with (+)-JQ1 validated BET bromodomain targeting as a promising therapeutic strategy in BL,²⁷ glioblastoma,³⁷ erythroleukemia,³⁸ high-risk acute lymphoblastic leukemia³⁹ and in aggressive B-cell lymphoma.⁴⁰ CPI203 is a BET inhibitor that has shown superior bioavailability with oral or intraperitoneal administration.^{28,29} We found MCL cells to be highly sensitive to CPI203 monotherapy, including bortezomib-resistant cell lines with increased MYC basal expression, where the compound achieved remarkable downregulation of the oncoprotein. We then demonstrated by both *in vitro* and *in vivo* approaches a meaningful synergistic effect of the CPI203–lenalidomide combination in MCL cases with intrinsic resistance to bortezomib, because of the capacity of CPI203 to facilitate lenalidomide-mediated downregulation of IRF4 and MYC.

Although the combination of lenalidomide with bortezomib is a rational one, given the clinical activity of both drugs in MCL, the combination of lenalidomide with such a BET protein bromodomain inhibitor is less obvious but may offer a glimpse into the potential opportunities that lenalidomide-based combinations hold for the treatment of patients with MCL refractory to bortezomib.

CONFLICT OF INTEREST

P Sandy and E Normant are employees with stock ownership in Constellation Pharmaceuticals, Inc. The remaining authors declare no conflict of interest.

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