The BET bromodomain inhibitor CPI203 improves lenalidomide and dexamethasone activity in *in vitro* and *in vivo* models of multiple myeloma by blockade of Ikaros and MYC signaling

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ABSTRACT

Most patients with multiple myeloma treated with current therapies, including immunomodulatory drugs, eventually develop relapsed/refractory disease. Clinical activity of lenalidomide relies on degradation of Ikaros and the consequent reduction in IRF4 expression, both required for myeloma cell survival and involved in the regulation of MYC transcription. Thus, we sought to determine the combinational effect of an MYC-interfering therapy with lenalidomide/dexamethasone. We analyzed the potential therapeutic effect of the combination of the BET bromodomain inhibitor CPI203 with the lenalidomide/dexamethasone regimen in myeloma cell lines. CPI203 exerted a dose-dependent cell growth inhibition in cell lines, indeed in lenalidomide/dexamethasone-resistant cells (median response at 0.5 μM: 65.4%), characterized by G1 cell cycle blockade and a concomitant inhibition of MYC and Ikaros signaling. These effects were potentiated by the addition of lenalidomide/dexamethasone. Results were validated in primary plasma cells from patients with multiple myeloma co-cultured with the mesenchymal stromal cell line stromaNKtert. Consistently, the drug combination evoked a 50% reduction in cell proliferation and correlated with basal Ikaros mRNA expression levels (P=0.04). Finally, in a SCID mouse xenotransplant model of myeloma, addition of CPI203 to lenalidomide/dexamethasone decreased tumor burden, evidenced by a lower glucose uptake and increase in the growth arrest marker GADD45B, with simultaneous downregulation of key transcription factors such as MYC, Ikaros and IRF4. Taken together, our data show that the combination of a BET bromodomain inhibitor with a lenalidomide-based regimen may represent a therapeutic approach to improve the response in relapsed/refractory patients with multiple myeloma, even in cases with suboptimal prior response to immunomodulatory drugs.

Introduction

Multiple myeloma (MM) is a hematologic malignancy characterized by neoplastic growth of bone marrow plasma cells. It constitutes almost 15% of all hematologic malignancies.[1,2] Virtually, all cases of MM are preceded by a pre-malignant state of clonal plasma cell known as monoclonal gammopathy of undetermined significance (MGUS).[3] The introduction of novel drugs, such as thalidomide, borte-

zomib and lenalidomide, has resulted in an improved response rate and progression-free survival (PFS).[4-6] Lenalidomide, like other immunomodulatory drugs (IMiDs), acts by modulating the substrate specificity of the CRL4-CRBN E3 ubiquitin ligase complex. This drug enhances the binding of Ikaros (IKZF1) and Aiolos (IKZF3) to the ubiquitin ligase complex, leading to a degradation of these two factors by the proteasome, thus inducing a reduction in their protein levels.[7-9] This, in turn, reduces MM cell survival.

Although the improvement achieved with new therapeutic approaches is clinically relevant, it is far from satisfactory as MM remains incurable, with a significant shortening of life-expectancy.[10] For this reason, research into new drugs to treat relapse or refractory MM patients constitutes a field of intense investigation. In this regard, bromodomain and extra-terminal (BET) inhibitors have emerged as promising molecules for the treatment of hematologic malignancies. BET family proteins (BRD2, BRD3 and BRD4) are chromatin adaptors, functionally linked to important pathways for cellular viability and cancer signaling. In particular, BRD4 has a direct role in the regulation of transcription of different genes involved in the cell cycle and transcription of oncogenes.[11] It has been demonstrated that BRD4 is highly expressed in dividing cells and has an important role in cell growth regulation.[12,13] Therefore, it is conceivable that its deregulation can influence cancer cell biology and the inhibition of BRD4 could effectively disrupt tumor growth.[14] Thus, BRD4 has been recently described as a therapeutic target for MM, among other hematologic diseases. The BET inhibitor (BETi) (+)-JQ1 selectively inhibits BRD4 by competitively binding to the acetyl-lysine recognition pocket of BET bromodomains from chromatin.[15,16] This displacement of BRD4 from chromatin leads to the inhibition of *MYC* transcription in a dose- and time-dependent manner. Although gene expression changes observed after BET-bromodomain inhibition are mainly dominated by the *MYC* transcriptome, BET inhibitors influence the expression of a more extensive assortment of nearly 3000 genes.[17,18] CPI203 is an analog of (+)-JQ1 with superior bioavailability *via* oral or intraperitoneal administration.[11] Moreover, the antitumoral effects of CPI203 are comparable, and even higher in some cases, than the effects of (+)-JQ1, both *in vitro* and *in vivo*.[11,19] Previous studies in MM showed the capacity of CPI203 to inhibit cell growth, even in cells resistant to bortezomib and melphalan.[18] CPI203 and bortezomib had a synergistic antiproliferative effect *in vitro*, where CPI203 causes a decrease in MYC expression levels sufficient to reduce proliferation and aggresome-mediated survival, yet permitting enough NOXA expression for bortezomib to potentiate apoptosis.[18] Accordingly, mantle cell lymphoma cells are also notably sensitive to CPI203 in bortezomib-resistant cells with increased MYC basal expression. In this scenario, CPI203 and lenalidomide synergistically inhibit the growth of bortezomib-resistant tumors.[20]

Malignant plasma cells in MM require Ikaros family zinc finger factor 1 (IKZF1) for their survival, which is therapeutically targeted by lenalidomide. As this protein is involved in the regulation of MYC transcription, our aim was to explore the activity of therapy with CPI203 targeting MYC in combination with a lenalidomide-based regimen in both *in vitro* and *in vivo* models of myeloma.

Table 1. Drug sensitivity and combination index (CI) of CPI203 and lenalidomide in MM cell lines.

Cell line	Lenalidomide sensitivity	CPI203 GI_{50} at 48h (μM)	CI values (CPI203 0.1 μM/Len 5 μM/Dex 0.1 μM)
ARP-1	Sensitive	0.16	0.082
JJN.3	Sensitive	0.08	0.514
U266	Sensitive	>1	0.331
MM.1S	Sensitive	>1	0.284
MM.1R	Sensitive	0.12	0.283
RPMI-8226	Resistant	>1	0.090
KMM.1	Resistant	0.23	0.379

MM: myltiple myeloma; h: hours; Len: lenalidomide; Dex: dexamethasone.

Methods

Cell lines and patient samples

Human myeloma cell lines ARP-1, JJN-3, U266, MM.1S, MM.1R, RMPI-8226 and KMM.1 were maintained in 10-15% FCS-supplemented RPMI-1640 medium (Thermo Fisher, Waltham, MA, USA).

Primary mononuclear cells from bone marrow aspiration of 9 patients [4 male (M)/5 female (F), median age: 63 years (range: 51-89)] with symptomatic MM were isolated by Ficoll/Hypaque sedimentation (GE Healthcare, Chalfont St Giles, UK). Ethical approval for this project, including patient informed consent, were granted following the guidelines of the Hospital Clinic Ethics Committee (IRB).

More detailed information is provided in the *Online Supplementary Methods*.

Cell proliferation assays

Myeloma cell lines ($5x10^4$ per well) were incubated with CPI203 (kindly provided by Constellation Pharmaceuticals, Cambridge, MA, USA) and/or lenalidomide (Selleck Chemicals LLC, Houston, TX, USA) plus dexamethasone (Merck, S.L., Darmstadt, Germany) at indicated doses in triplicates. MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich, St Louis, MO, USA) was used to evaluate the effect of the drugs on cell proliferation.

Primary cells were labeled with CellTracker™ Red CMPTX dye (Thermo Fisher) following the manufacturer's protocol and co-cultured with the mesenchymal stromal cell line stromaNKtert in the presence of 10 ng/mL IL-6 (RnD Systems, Minneapolis, MN, USA). Cell proliferation was analyzed in an Attune acoustic focusing cytometer using Attune software (Thermo Fisher).

Gene expression profiling and gene set enrichment analysis

RNA was analyzed on Affymetrix Human Genome U219 arrays. Gene set enrichment analysis (GSEA) v.2.0 (Broad Institute at MIT, Boston, USA; *http://www.broadinstitute.org/gsea/*) was used to identify gene signatures, interrogating C2CP and C3TFT gene sets from the Molecular Signature Database v.2.5 and experimentally-derived custom gene sets related to Ikaros.[21] The microarray data have been deposited in the NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE87403.

Xenograft mouse model

SCID mice (Charles River Laboratories, L'Arbresle, France) were inoculated subcutaneously with $1.2x10^7$ cells of RPMI-8226 cell line. Mice were randomly assigned into cohorts of 5 mice each and received either a twice-daily dose of CPI203 (2.5 mg/kg) for two weeks, or a daily dose of lenalidomide (25 mg/kg) plus twice weekly dexamethasone (1 mg/kg), or the combination of both, or an equal volume of vehicle. Twenty-one days post-cell inocula-

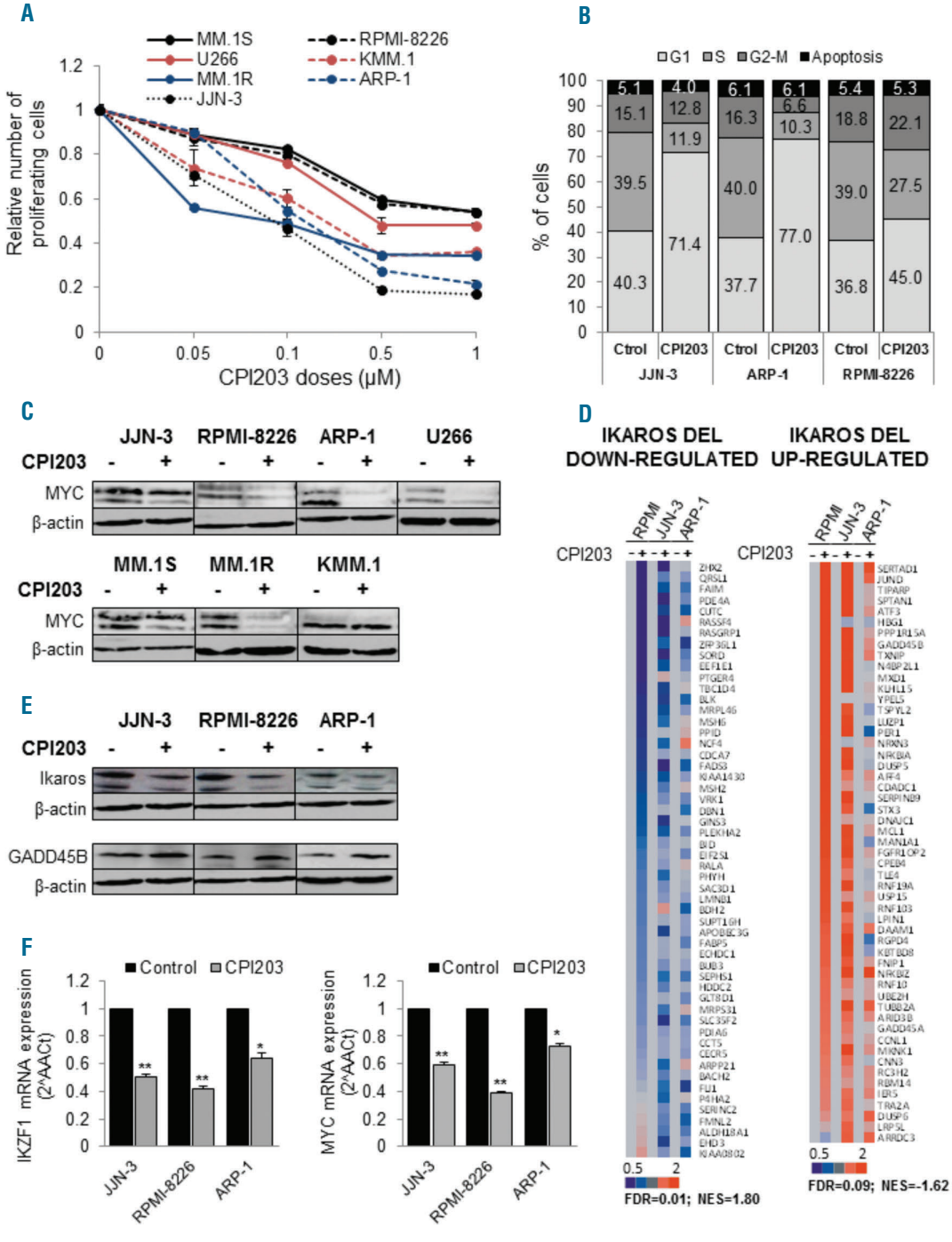

Figure 1. Characterization of CPI203 effect in multiple myeloma cell lines. (A) A set of seven myeloma cell lines were exposed to increasing concentrations of CPI203 for 48 hours (h). The relative number of proliferating cells was analyzed by MTT assay. Results are represented as mean±Standard Error of Mean (SEM) of triplicate assays. (B) JJN-3, RPMI-8226 and ARP-1 cells were treated for 24 h with 0.1 μM CPI203 and cell cycle fractions were determined by flow cytometry of propidium iodide-labeled nuclei. (C) MYC protein levels were analyzed by Western blot after 0.1 μM CPI203 treatment (48 h) in the 7 cell lines; β-actin was used as loading control. (D) Heatmaps of the leading edges of IKAROS-related gene sets identified as enriched by GSEA in cells treated with CPI203 (6 h) *versus* control. Threshold FDR=0.01 and NES=1.80 and FDR=0.09 and NES=-1.62 for gene sets "Ikaros del down-regulated" and "Ikaros del up-regulated", respectively. (E) Changes in the expression of the selected genes (Ikaros and GADD45B) were confirmed by Western blot in three representative myeloma cell lines after 24 h of treatment. (F) *IKZF1* and *MYC* mRNA expression in three representative cell lines tested after 6 h of treatment with CPI203 (0.1 μM). Results are referred to the untreated control and GUSB was used as endogenous control. Data are shown as mean±SEM. *t*-test was performed with reference to the control. **P*<0.05, ***P*<0.01.

tion, intratumoral glucose uptake was evaluated with an Odyssey infra-red scanner (Li-Cor, Lincoln, NE, USA) in mice previously injected with an IRDye 800CW 2-deoxyglucose probe (Li-Cor). Animals were then sacrificed according to institutional guidelines and tumor xenografts were isolated. Paraffin-embedded tumor samples were subjected to immunohistochemical staining using primary antibodies against Ikaros, MYC, IRF4, GADD45B and pH3 and evaluated with an Olympus DP70 microscope by means of a 20x/0.75 NA objective and DPManager software v.2.1.1 (Olympus).

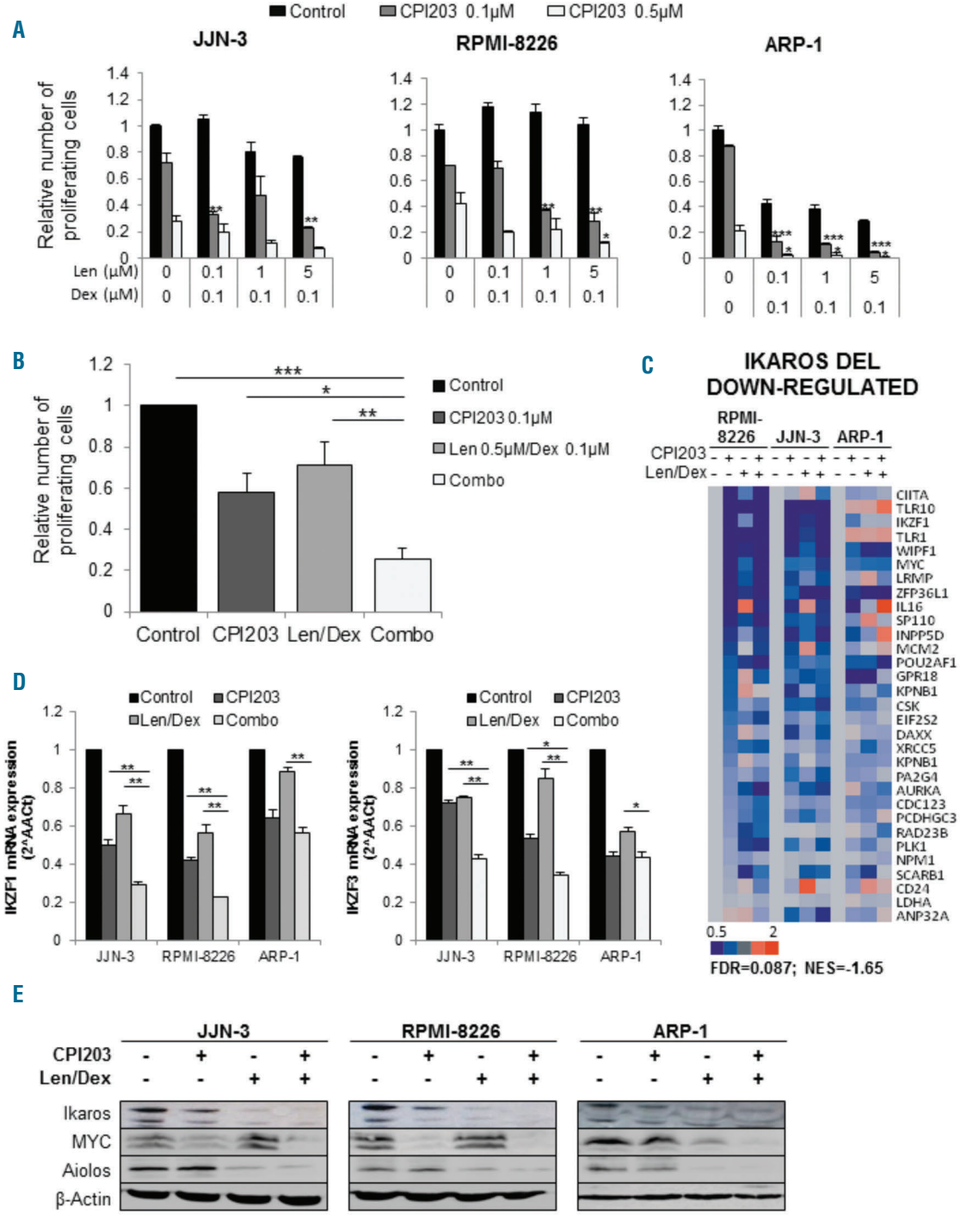

Figure 2. Synergistic antiproliferative effect due to modulation of MYC and Ikaros by CPI203/Len/Dex. (A) JJN-3, RPMI-8226 and ARP-1 were treated with increasing doses of CPI203 and/or Len/Dex and the drug cytostatic effect was analyzed by MTT proliferation assay after 48 hours (h). (B) Relative proliferation of 7 myeloma cell lines after 48 h of treatment with CPI203 (0.1 μM), Len/Dex (5 μM/100 nM) and the 3-drug combination. Data are shown as mean±Standard Error of Mean (SEM). One-way ANOVA test was performed; *P*=0.0003 was considered statistically significant. **P*<0.05, ***P*<0.01,****P*<0.001. (C) Heatmap of the leading edge of Ikaros-related gene sets identified as negatively enriched in the combo by GSEA using an increasing profile analysis. Threshold FDR=0.087 and NES=-1.65. (D) *IKZF1* and *IKZF3* mRNA expression in all myeloma cell lines tested after 6-h treatment with CPI203 (0.1 μM) and/or Len/Dex (5 μM/100 nM). Results are referred to the untreated control and GUSB was used as endogenous control. (E) Changes in the expression of the selected factors (Ikaros, MYC and Aiolos) after 24 h of CPI203 and/or Len/Dex treatment were confirmed by Western blot in three cell lines. Data are shown as mean±SEM. *t*-test was performed comparing CPI203 and Len/Dex as single agents with combo. **P*<0.05, ***P*<0.01, ****P*<0.001.

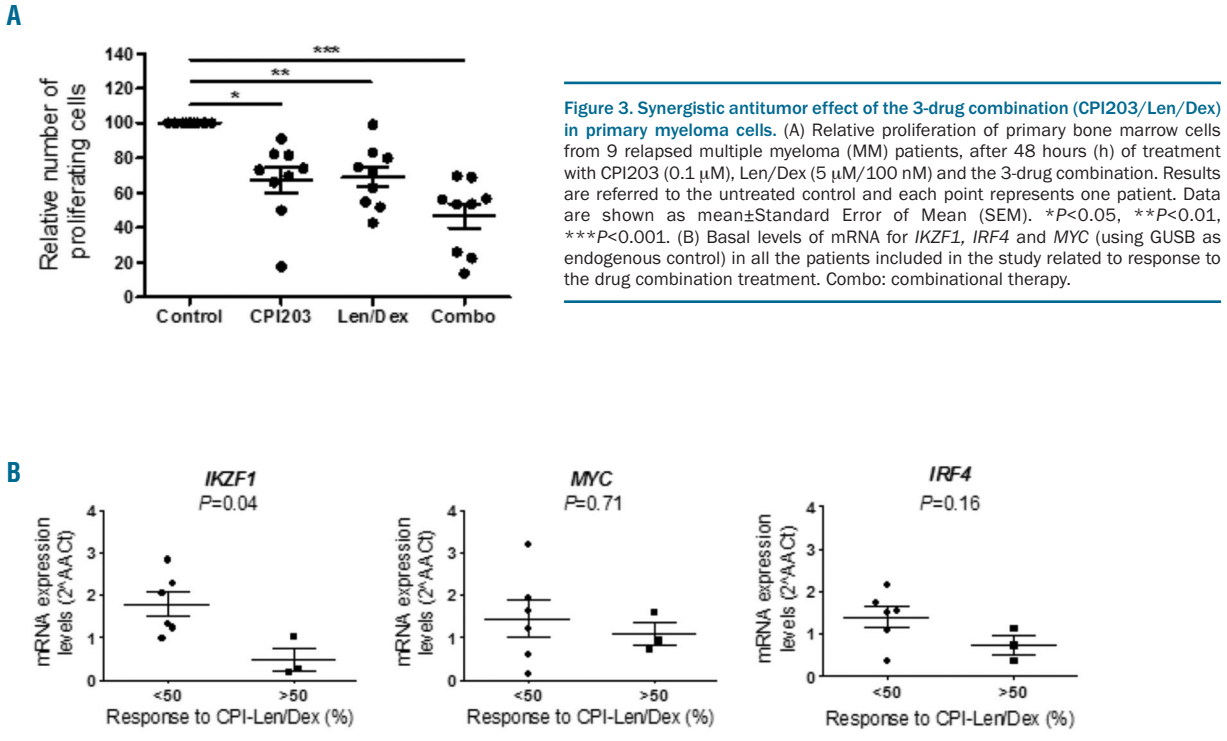

Figure 3. Synergistic antitumor effect of the 3-drug combination (CPI203/Len/Dex) in primary myeloma cells. (A) Relative proliferation of primary bone marrow cells from 9 relapsed multiple myeloma (MM) patients, after 48 hours (h) of treatment with CPI203 (0.1 μM), Len/Dex (5 μM/100 nM) and the 3-drug combination. Results are referred to the untreated control and each point represents one patient. Data are shown as mean±Standard Error of Mean (SEM). **P*<0.05, ***P*<0.01, ****P*<0.001. (B) Basal levels of mRNA for *IKZF1, IRF4* and *MYC* (using GUSB as endogenous control) in all the patients included in the study related to response to the drug combination treatment. Combo: combinational therapy.

Statistical analysis

All statistical analyses were performed using GraphPad software 5.0 (GraphPad Software Inc., San Diego, CA, USA). The comparisons between all analyzed groups were evaluated with a Kruskal-Wallis test and the comparisons between two groups were analyzed with Student *t*-test or non-parametric Mann-Whitney test. $P<0.05$ was considered statistically significant; data are represented as mean±Standard Error of Mean (SEM) of 3 independent experiments (**P*<0.05, ***P*<0.01, ****P*<0.001).

Results

Anti-myeloma activity of CPI203 is independent of cell sensitivity to lenalidomide and involves MYC and IKZF1 downregulation

CPI203 has recently been shown to exert a significant antitumor activity in the low micromolar range of concentration in MM cell lines, irrespective of the primary response to the proteasome inhibitor bortezomib or to the alkylating agent melphalan.[18] To investigate the activity of the compound with regards to the response to a lenalidomide-based therapy, a panel of 5 lenalidomide-responsive and 2 lenalidomide-resistant cell lines was exposed for 48 hours (h) to CPI203 doses ranging from 0.05 to 1 μM and cell viability was measured by MTT assay. The compound exerted a dose-dependent inhibition of proliferation in all the MM cell lines tested, with the optimal reduction in cell proliferation achieved at the 0.5 μM dose (median response: 65.4%, range: 40-81%) (Figure 1A), while in the most sensitive cell lines (MM.1R, JJN-3 and KMM-1) the GI_{50} value decreased to below 100 nM (Table 1). Of interest, cell response to the compound was independent of primary response to lenalidomide, as resistant cells showed a similar response to the sensitive ones (median response: 54.1%, range: 43-66%). In agreement with previous reports, the compound failed to evoke apoptotic cell death in MM cells since its activity mainly related to a significant blockade of the cell cycle at the G1 phase (mean increase of apoptotic cells: 15.1%) in the three representative cell lines analyzed: JJN-3 (32%), ARP-1 (42%) and RPMI-8226 (9%) (Figure 1B). This effect was accompanied by a decrease in MYC protein levels in all the cell lines, although a strict correlation could not be observed with the efficacy of the compound (Figure 1C), thus arguing in favor of a role for additional mechanism(s). To better characterize the main factors involved in MM response to CPI203, we then performed gene expression profiling (GEP) analysis with the three cell lines used previously, either untreated or treated for 6 h with 0.1 μM CPI203. We performed GSEA using well-defined and previously described gene signatures.[20] As expected, there was an enrichment of genes up-regulated by MYC and genes down-regulated by the transcription factor BLIMP1 in the control cells when compared to CPI203-treated samples (*Online Supplementary Table S1* and *Online Supplementary Figure S1*). It is noteworthy that among the different proliferation-associated gene sets analyzed there was a simultaneous, marked upregulation of Ikaros-repressed genes and downregulation of Ikaros-induced genes in cells exposed to the BET inhibitor (Figure 1D and *Online Supplementary Table S1*). As Ikaros has been shown to be a crucial regulator of the G1 to S transition of the cell cycle,[22] we checked for the presence of G1 regulatory factors among the top 10 genes regulated by CPI203 and included in the two Ikaros gene sets considered; we identified GADD45B, a well-known negative regulator of cell cycle progression.[23] Consistently, we observed a concomitant downregulation of Ikaros (mean reduction: 70.4%; range: 55.1-82.2%) and increase in GADD45B protein levels (mean increment: 45.2%; range: 34.5-58.9%) after 24 h of treatment with 0.1 μM CPI203 in the three representative cell lines (Figure 1E). As expected, CPI203 induced a decrease in MYC and IKZF1 mRNA levels (mean decrease:

40-50%) (Figure 1F); this transcriptional repression could explain the upregulation of GADD45B.

CPI203 synergistically increases lenalidomide-induced blockade of MYC and Ikaros signaling *in vitro*

Reduction in Ikaros protein levels has been well documented and related to cereblon-dependent proteasomal degradation of the transcription factor in MM cells after treatment with lenalidomide.[7,9,24] To determine if transcriptional repression of the corresponding gene by means of CPI203 could offer an improvement in lenalidomide antitumor activity, we treated our panel of 7 MM cell lines with standard doses of the Len-Dex regimen, in the presence or the absence of the BET inhibitor at the two optimal doses previously described (0.1 and 0.5 μM). We had previously tested all three drugs individually, and the combination of lenalidomide and/or dexamethasone with CPI203 in four MM cell lines (MM1.S, KMM.1, U266 and RPMI-8226). In this preliminary study, we observed that neither lenalidomide nor dexamethasone as single agent had a combinational effect with the BRD4 inhibitor (*Online Supplementary Figure S2)*. In contrast, cell treatment with the Len/Dex combination, which corresponds to the regimen proposed to patients with MM in clinical practice, allowed a remarkable improvement of CPI203 activity with both concentrations of the drug in all seven cell lines tested (Figure 2A and *Online Supplementary Figure S3*). In order to better evaluate the co-operation between the two drugs, we calculated the Combination index (CI) in each cell line, based on the Chou-Talalay method. The best combinational activity was achieved when combining the 0.1 μM dose of CPI203 with the treatment by lenalidomide (5 μM) and dexamethasone (0.1 μM), obtaining CI values ranging from 0.082 to 0.514 (mean: 0.280) (Table 1). As CI values between 0.3 and 0.7 indicate a synergistic effect, and values between 0.1 and 0.3 suggest a strong synergism between the two drugs of interest, our results suggest a high synergistic effect of the 3-drug combination. These doses were those used in all the validation experiments. At these doses, single agent CPI203 induced a 42.7% reduction (range: 13-74%; $P<0.05$), which achieved up to 76.1% reduction (range: 53-96%; $P<0.001$) when combined with Len/Dex treatment (Figure 2B). Although neither CPI203 nor Len/Dex individually caused a significant increase in cell apoptosis, the combination of these drugs resulted in a mean relative increase in the number of apoptotic cells of 37.9%. In order to validate the main gene signatures involved in this effect, we ran a new GSEA analysis using an increasing profile mode, comparing control cells with CPI203-treated and CPI203/Len/Dex-treated samples, in the same conditions as described previously. As shown in *Online Supplementary Table S2*, and as exemplified in Figure 2C and *Online Supplementary Figure S4*, gene sets related to MYC function as transcriptional regulator or with plasma cell differentiation (i.e. BLIMP-1 and IRF4-dependent gene sets) were significantly more affected by the CPI203/Len/Dex combination than by CPI203 alone. Of special interest, the group of genes positively regulated by Ikaros was also disrupted by the combination treatment (Figure 2C). Western blot analysis confirmed the increasing reduction in Ikaros and MYC protein levels with the sequential addition of the different drugs, being both factors dramatically decreased in the CPI203/Len/Dex drug combination treated cells (Figure 2E). As expected, while cells exposed to single agent CPI203 or CPI203/Len/Dex combination harbored a 40-50% reduction in *IKZF1* mRNA levels, respectively, Len/Dex treatment alone did not significantly affect Ikaros transcript levels, confirming a post-transcriptional regulation of Ikaros expression (Figure 2D). As previously described, lenalidomide also induces the degradation of Aiolos *via* interaction with CRBN. Thus we analyzed both mRNA expression and protein levels of this factor upon myeloma cell exposure to the drug. The modulation of *IKZF3* mRNA after CPI203 and/or Len/Dex treatment was very similar to that seen with IKZF1 (Figure 2D). However, we observed that CPI203, as single agent, had no effect on Aiolos protein expression, and downregulation of this protein in cells receiving the 3-drug combination was mainly due to lenalidomide activity (Figure 2E).

BET bromodomain inhibition increases Len/Dex efficacy in MM primary cultures

To validate the above results in MM primary samples *ex vivo*, bone marrow aspirates from 9 symptomatic MM patients with high contents in tumor cells (mean percentage $CD38^+$ cells: 60%) were cultured for 48 h upon a monolayer of the mesenchymal stromal cell line stromaNKtert[25] plus IL-6,[26] and in the presence or the absence of CPI203 (0.1 μM) and/or lenalidomide (5 μM)/dexamethasone (0.1 μM) combination. In the absence of drug, a subset of primary $CD38^+$ cells underwent cell cycle entry and proliferation as evaluated by cell labeling with a CellTracker dye (mean: 15%). When referred to control cells, CPI203- and Len/Dex-exposed samples showed a 33% and 30% reduction in stroma-mediated cell proliferation, respectively, while this effect was increased up to 53% in the case of the drug combination (*$P<0.05$, **$P<0.01$, ***$P<0.001$) (Figure 3A). Of special interest, in this set of primary samples there was a significant correlation between IKZF1 mRNA levels and cell response to the CPI203/Len/Dex combination, as those cases with lower levels of Ikaros showed a higher response to this treatment ($P=0.04$) (Figure 3B). Such a correlation was not found when considering IRF4 or MYC expression (Figure 3B); thus highlighting the crucial role of Ikaros towards BETi/Len/Dex combinational activity in primary MM cells.

The CPI203/Len/Dex combination inhibits MM tumor growth *in vivo*

To further characterize the co-operative role of CPI203 and Len/Dex *in vivo*, SCID mice inoculated with RPMI-8226 cells were randomly assigned into three treatment groups (CPI203 2.5 mg/kg BID, lenalidomide 50 mg/kg daily plus dexamethasone 1 mg/kg twice weekly and combination) and the vehicle-treated groups. While CPI203 and Len/Dex achieved a 62% and 61% reduction in tumor volume ($P=0.031$ and $P=0.023$, respectively), when compared to the vehicle group, the combination of BETi and Len/Dex resulted in complete arrest of tumor growth in mice receiving the CPI203/Len/Dex combination ($P=0.0012$) (Figure 4A). Accordingly, tumor glucose uptake was reduced to 47% and 45% in animals treated with CPI203 and Len/Dex, respectively, while the combination therapy resulted in a 64% reduction (Figure 4B). Immunohistochemical analysis of the corresponding tumors confirmed an additional decrease in the mitotic index as shown by phospho-histone H3 staining, together with the almost complete disappearance of MYC-, IRF4-

and Ikaros-positive cells, and a remarkable accumulation of GADD45B-expressing cells in the group receiving the drug combination (Figure 4C). These results confirmed our *in vitro* data, showing that the combination of the BET inhibitor CPI203 with Len/Dex augments the antitumor properties of each single agent, and results in the abrogation of Ikaros and MYC signaling and consequent blockade of tumor growth.

Discussion

Constitutive activation of MYC signaling is detected in more than 60% of patient-derived myeloma cells[27] and can be involved in the pathogenesis of MM through different mechanisms related to the progression from early stages, such as MGUS, to symptomatic disease. One of the most common somatic genomic aberrations in early-

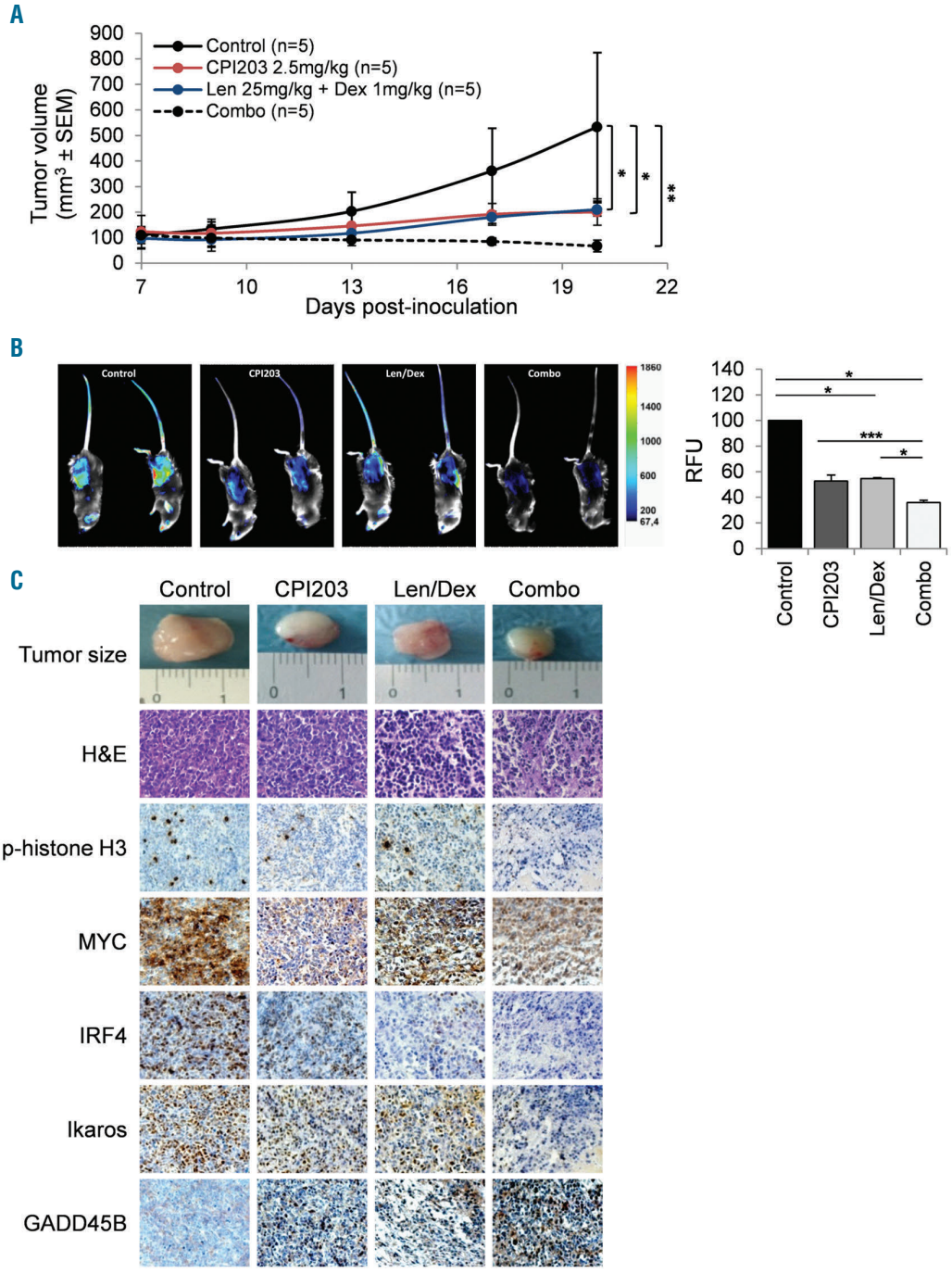

Figure 4. CPI203 plus Len/Dex synergistically inhibits tumor growth in multiple myeloma (MM) mouse model. (A) Mice were inoculated with 1.2x10^7 RPMI-8226 cells and treatment began at day (d)7 post-cell inoculation. Evolution of tumor burden during the treatment; volumes were recorded every 3-4 days by external calipers. (B) Intratumoral glucose uptake images obtained with an Odyssey infra-red scanner and their corresponding fluorescence quantifications from representative mice at the day of sacrifice. Data are shown as mean±Standard Error of Mean (SEM). *P<0.05, **P<0.01, ***P=0.001. (C) Tumor size (mm) and immunohistochemical staining of hematoxylin and eosin (H&E), p-histone H3, GADD45B, Ikaros, MYC and IRF4 in consecutive tissue sections from tumors after two weeks of indicated treatment.

and late-stage MM is rearrangement or translocation of MYC.[28] Moreover, oncogenic super enhancers can recruit the BET family and consequently augment the aberrant MYC expression.[29] Given that BET bromodomain inhibition has previously been shown to disrupt MYC signaling among other pathways in different hematologic cancers, we sought to determine if the BETi might represent a new therapeutic option for MM. In this study, we have demonstrated that the BRD4 selective inhibitor CPI203 could be used either as a single agent (considering its remarkable activity as monotherapy *in vitro* and *in vivo*) or in combination with standard therapies, as shown previously with the proteasome inhibitor bortezomib.[18] Interestingly, as observed in mantle cell lymphoma,[20] the synergistic activity of CPI203 with bortezomib in MM also overcame resistance to this proteasome inhibitor. Here, we followed a similar design by including two cell lines with reduced sensitivity to lenalidomide and demonstrated by *in vitro* and *in vivo* approaches the antitumoral activity of this molecule, as well as its capacity to enhance MM response to the immunomodulatory drug, thus highlighting possible therapeutic implications. Moreover, our results are in agreement with a previous publication in primary effusion lymphoma which demonstrated a synergistic effect on cytotoxicity between IMIDs and BDR4 inhibitors.[30]

While, despite the advances in the management of the disease, MM remains incurable, strategies based on the combination of IMiDs with other agents have improved the prognosis of these patients. For example, the VTD (bortezomib, thalidomide and dexamethasone) combination is a highly effective induction regimen prior to autologous stem cell transplantation (ASCT) to treat patients with standard- and high-risk MM, although 15% of patients fail to respond.[6] Moreover, the duration of responses is limited and nearly all patients relapse and require salvage chemotherapy. In this sense, rescue therapy with Len/Dex is effective in increasing the response rate, the time to progression and overall survival in patients with relapsed or refractory MM,[31-33] being an established treatment option for this group of patients. Len/Dex also constitutes the back-bone of combination therapy with newer agents, such as proteasome inhibitors or monoclonal antibodies.[34,35] According to our *in vivo* results, the addition of CPI203 to Len/Dex allowed for an almost complete and prolonged inhibition of tumor growth, where probably dexamethasone plays a crucial role (either direct or indirectly) as seen when used in patients. In this sense, this 3-drug strategy may improve responses compared to the effects of combining new-generation IMiDs with dexamethasone.[6,36-38] Thus, following the current phase I clinical trials testing the BET bromodomain inhibitor in different hematologic malignancies, including patients with previously treated MM (*clinicaltrials.gov identifier: 02157636*), it would be interesting to design phase I/II clinical trials including this triple drug combination in relapsed/refractory (R/R) MM patients.

Mechanistically, lenalidomide is known to bind cereblon, with the subsequent activation of the E3-ubiquitin ligase activity that results in the degradation of key transcription factors like Ikaros. Moreover, lenalidomide indirectly inhibits IRF4 expression, mainly through downregulation of Ikaros and Aiolos.[9] CPI203 has been reported to cause significant decreases in MYC expression, which may be sufficient to reduce proliferation and aggresome-mediated survival.[18] Using a GEP approach, we have identified a new role for BETi in the regulation of Ikaros-regulated factors at both the transcript and protein levels, in addition to the established role of BETi in the MYC/IRF4 signaling axis in MM. Among this group of genes, the expression of the negative regulator of cell cycle progression, GADD45B, tightly correlated with the G1 cell cycle blockade observed in MM cell lines upon CPI203 exposure. In agreement with these data, the combination of CPI203 with Len/Dex therapy induced a synergistic effect on proliferation in all the MM cell lines and a co-operative effect on primary cases analyzed, reaching between 50% and 80% global antiproliferative activity, in close correlation with a decrease in Ikaros protein in the case of the cell lines, or in basal Ikaros transcript levels in the cases of MM primary cultures. The identification of Ikaros-dependent signaling as a constant parameter involved in CPI203/Len/Dex response may be of special interest, as the identification of potential predictive response biomarkers may allow an individualized selection of patients to receive these specific treatments. In this regard, an association has been described between cereblon expression and response to lenalidomide and dexamethasone in patients with MM.[39,40] Specifically, response and survival in patients with MM treated with lenalidomide improved when protein levels of cereblon were higher. In our study, higher basal levels of Ikaros in patients correlated with the poorest *in vitro* responses to the drug combination, in accordance with the concept that overexpression of Ikaros in MM cells could induce resistance to lenalidomide.[8,9] Nevertheless, further studies in larger series of patients evaluating Ikaros expression as a potential marker of response to BETi-based regimens would be required to confirm these first observations.

In summary, following our initial aim to explore the combinational activity of therapy targeting MYC based on CPI203-mediated bromodomain inhibition with a lenalidomide-based regimen in malignant plasma cells, we demonstrate here that CPI203 is as efficient *in vitro* and *in vivo* as the approved Len/Dex therapy at the concentrations currently used in clinical settings to treat patients with R/R MM. More interestingly, we show a constant and rationally-based co-operation between CPI203 and Len/Dex therapy in both *in vitro* and *in vivo* models of MM. The combination of BET bromodomain inhibitors with the Len/Dex therapy is a logistically feasible approach, and could be considered as an option to improve the response in R/R patients with MM, even in cases with suboptimal prior response to IMIDs.

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