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The anti-mitotic agents PTC-028 and PTC596 display potent activity in pre-clinical models of multiple myeloma but challenge the role of *BMI-1* as an essential tumour gene

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Summary

Future progress in the treatment of multiple myeloma (MM) requires both the characterisation of key drivers of the disease and novel, innovative approaches to tackle these vulnerabilities. The present study focussed on the pre-clinical evaluation of a novel drug class, BMI-1 modulators, in MM. We demonstrate potent activity of PTC-028 and PTC596 in a comprehensive set of *in vitro* and *in vivo* models, including models of drug resistance and stromal support. Treatment of MM cells with PTC-028 and PTC596 downregulated BMI-1 protein levels, which was found to correlate with drug activity. Surprisingly, *BMI-1* was dispensable for the activity of BMI-1 modulators and MM cell growth. Our data rather point to mitotic arrest accompanied by myeloid cell leukaemia-1 (MCL-1) loss as key anti-MM mechanisms and reveal impaired MYC and AKT signalling activity due to BMI-1 modulator treatment. Moreover, we observed a complete eradication of MM after PTC596 treatment in the 5TGM.1 *in vivo* model and define epigenetic compounds and B cell leukaemia/lymphoma 2 homology domain 3 (BH3) mimetics as promising combination partners. These results bring into question the postulated role of *BMI-1* as an essential MM gene and confirm BMI-1 modulators as potent anti-mitotic agents with encouraging pre-clinical activity that supports their rapid translation into clinical trials.

Keywords: BMI-1, myeloma, pre-clinical, PTC-028, PTC596.

Introduction

Although intensified research efforts throughout the last decade have led to a striking extension in the clinical armamentarium to combat multiple myeloma (MM), MM remains a difficult to treat disease, particularly in the relapsed/refractory setting.[1] This is at least in part attributed to the fact that several known drivers of MM cannot be effectively targeted. Although innovative strategies such as proteolysis targeting chimeras (PROTACs) raise hope to hit several neoplastic key factors in the near future, these novel drugs are currently only available for a limited number of targets (most notably bromodomain-containing protein 4 [BRD4]).[2] Many other established drivers of MM, and human malignancies in general, remain currently 'undruggable'.

The polycomb group protein BMI-1 represents a prominent intrinsic driver without a suitable clinical grade inhibitor. BMI-1 was discovered as an accelerator of MYC-driven lymphomas[3] and subsequent studies revealed its pleiotropic function in several cellular processes (e.g. cell cycle, DNA damage, apoptosis, senescence, and self-renewal of stem cells).[4] BMI-1 was also shown to play a central role in cancer stem cell growth and survival in various neoplasia.[4-7] Mechanistically, BMI-1 acts as part of the polycomb repressive complex 1 (PRC1), which represses gene expression via mono-ubiquitination of histone H2A at lysine 119. This process is tightly coupled with PRC2-mediated histone H3 lysine 27 (H3K27)-trimethylation and initiates the recruitment of DNA methyltransferases (DNMTs) for stable silencing of distinct chromatin regions.[8] More recent insights challenged this hierarchical model by revealing PRC2-independent recruitment of PRC1, which led to the classification of the diverse PRC1 functions into canonical and non-canonical mechanisms.[9] Regarding myeloma, BMI-1 overexpression was

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linked to the pathogenesis of MM more than a decade ago.[10] Silencing of *BMI-1* with short-hairpin RNAs (shRNAs) was shown to impair the outgrowth of myeloma cells *in vitro* and *in vivo* via modulation of B-cell leukaemia/lymphoma 2 (Bcl-2) family members (i.e. Bim and Bax).[11,12] These findings established its role as an essential MM gene.

We and others recently confirmed BMI-1 overexpression as a hallmark of MM and its putative role as a drug target using the small molecule inhibitor PTC-209.[13,14] Based on these observations, in the present study, we explored the anti-myeloma efficacy of the first-in-class BMI-1 modulators PTC-028 and PTC596. BMI-1 modulators have been described to target BMI-1 in an indirect manner via inhibition of the anaphase promoting complex $(APC/C)^{CDC20}$ leading to persistent cyclin-dependent kinase (CDK)1/2 activity and BMI-1 hyper-phosphorylation, as well as reduced PRC1 activity.[15] However, recent data questioned the role of BMI-1 for the activity of PTC596 and rather pointed to BMI-1 modulators as potent microtubule polymerisation inhibitors.[16] Importantly, these compounds are active at low nanomolar concentrations and differ from other agents of this drug class by the lack of neurotoxicity, making them interesting candidates for clinical programmes, which is underlined by several early phase trials that are currently examining the potency of the lead compound PTC596 in solid tumours (ClinicalTrials.gov Identifiers: NCT02404480, NCT03206645, NCT03605550, NCT03761095).

Patients and Methods

Patient samples

Primary MM cells were purified from patients with MM undergoing routine bone marrow (BM) aspiration at the Department of Medicine I (Wihelminenspital, Vienna). Written informed consent for the use of material for scientific studies was obtained from these patients according to institutional guidelines. Mononuclear cells were isolated by Ficoll-Hypaque density sedimentation and MM cells were purified using the EasySepTM Human CD138 Positive Selection Kit (Stemcell Technologies Inc., Cologne, Germany) and used for co-culture experiments as previously described.[17]

Flow cytometry

The measurement of apoptosis by Annexin V/7-amino-actinomycin (AAD) staining, JC-1 assay, detection of cleaved poly-[ADP-ribose]-polymerase (PARP), as well as cell cycle analysis have been previously described.[14,17] For the evaluation of co-culture experiments MM cells were distinguished from BM stromal cell (BMSC) telomerase reverse transcriptase $(TERT)^{+}$ cells via green fluorescent protein (GFP) expression in stromal cells.[18] Intracellular staining of BMI-1 (phycoerthyrin [PE] mouse anti-human BMI-1 antibody #562528; PE mouse immunoglobulin (Ig)G1κ isotype control: #554680) was performed using the BD Transcription Factor Buffer Set according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). BMI-1 negativity by flow cytometry was defined by the concurrent use of the isotype control and displayed as relative shift after BMI-1 modulator treatment $(\frac{BMI1\ negative\ cells\ post\ treatment}{BMI1\ negative\ cells\ without\ drug\ exposure})$. Histone 3 phospho-Serine-28 levels were analysed with a commercially available antibody (BD Biosciences) according to the manufacturer's instructions. Analyses were performed on a FACScan and FACS Canto II (BD Biosciences).

Cytotoxicity, colony formation and cell growth assays

Viability and colony formation was determined using Cell Counting Kit 8 (GERBU Biotechnik, Heidelberg, Germany) and MethoCult Classic medium (StemCell Technologies, Cologne, Germany) as previously described.[14] For the evaluation of MM cell growth post-transduction with BMI-1 shRNAs or overexpression vectors, MM cells (50 000/ml) were seeded in 24-well plates and cell numbers were determined over a period of 8 days using a Z2 Cell and Particle Counter (Beckman Coulter, Brea, CA, USA). In brief, cells were re-suspended at the indicated days and 20 μl cell suspension per well was used for the determination of total cell numbers.

In vivo *studies*

In vivo experiments were performed using the 5TGM.1 murine model of myeloma as previously described.[19] These experiments were approved by the ethical committee for animal experiments of the University of Liége (ULg license #16-1897). Treatment was initiated 1 day after the injection of myeloma cells (9-week-old female mice were injected i.v. with $5{\cdot}0 \times 10^5$ 5TGM.1GFP + cells). After tumour inoculation, the mice were followed and examined on a daily schedule, evaluating (i) their mobility and movements, (ii) their posture and global attitude, (iii) social behavior and (iv) their pilosity (Table S5). Because tumour take is homogenous, the number of mice to be included can be reduced to five mice per group. Mice were randomly divided into a naïve (healthy controls), a vehicle-treated (5TGM.1 myeloma-bearing mice receiving vehicle solution) and a treatment group (5TGM.1 myeloma-bearing mice receiving PTC596). PTC596 was dissolved in 0·5% HP-methylcellulose and 0·1% Tween-80 and administered by oral gavage at either 7·5 mg/kg/biweekly, 15 mg/kg/biweekly, or 30 mg/kg/weekly. When mice showed signs of active myeloma, that is, paraplegia, all mice within a cohort were humanely killed. At the end of the treatment period (day 29 in this study) the efficacy of PTC596 was determined by quantifying BM myeloma cell infiltration via flow cytometry (GFP + 5TGM.1) and comparing IgG serum concentrations. This experiment was repeated twice.

Statistical analysis

A two-tailed unpaired *t*-test was performed for the comparison of two means and Kruskal–Wallis test followed by a Dunn's test for multiple comparisons for the comparison of more than two means by GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). P values $< 0{\cdot}05$ were considered to be statistically significant. Drug combinations were analysed with CompuSyn software (www.combosyn.com). Combination index (CI) values of $< 0{\cdot}8$, 0·8–1·2, and >1·2 were interpreted as synergistic, additive, and antagonistic drug activity, respectively. Dependency scores for BMI-1, MYC and interferon regulatory factor 4 (IRF4) were obtained from the depmap project webportal of the BroadInstitute (Q4, 2018 release). All graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates, unless otherwise indicated.

A detailed description of additional materials and methods used is provided in the *Online Supplement* of the manuscript.

Results

BMI-1 modulators target BMI-1 and impair MM cell viability

Given the non-direct impact of BMI-1 modulators on *BMI-1* function and protein levels, we first investigated whether PTC-028 and PTC596 target BMI-1 in myeloma cells. Both compounds induced a time-dependent reduction of BMI-1 protein levels (Fig 1A). Dose-response curves demonstrated a significant impact on MM cell (but not BMSC) viability at low nanomolar concentrations (Fig 1B). The median half-maximal inhibitory concentration (IC_{50}) levels 96 h after treatment (PTC-028: 39·6 nM, range: 11·7–102·3; PTC596: 57·2 nM; range: 16·9–137·2 nM) were significantly lower compared to the previously reported BMI-1 inhibitor PTC-209 (median IC_{50} 680 nM, range: 207–5676, $P < 0{\cdot}05$) (Fig S1). Similar potency was observed in ixazomib- and carfilzomib-resistant cell line variants (Fig S2). Importantly, the 96-h IC_{50} levels correlated with the accumulation of BMI-1-negative cells 24 h after treatment. Five out of seven MM cell lines analysed displayed a relative shift of BMI-1-negative cells at the single cell level (flow cytometry) 24 h after treatment. The median (range) percentage of BMI-1-negative cells increased from 32·2 (21·1–61·8)% in untreated cells to 43·3 (27·4–54·6)% and 40·2 (26·9–58·7)% in PTC-028- and PTC596-treated cells, respectively. This suggests that early fluctuations of BMI-1 protein levels in a subfraction of tumour cells could serve as a predictive biomarker in future clinical programmes (Fig 1C). No association was seen between baseline BMI-1 mRNA or protein expression and IC_{50} levels (not shown).

BMI-1 is dispensable for the activity of BMI-1 modulators and MM cell growth

To analyse whether BMI-1 degradation is responsible for the activity of PTC-028 and PTC596, we generated BMI-1 overexpressing cell lines. There was no difference between the growth of control (EGFP overexpression) and BMI-1 overexpressing cells (Figs S3A). In addition, we observed no rescue effect against BMI-1 modulators in cell lines with BMI-1 overexpression, suggesting that other mechanisms are responsible for their potent anti-MM activity (Figs S3B).

To strengthen this finding, we generated stable *BMI-1* knockdown variants using five BMI-1 shRNAs in four human multiple myeloma cell lines (HMCLs) with distinct genomic background (Fig 2A). Again, PTC-028 and PTC596 activity did not differ between cells expressing scrambled or *BMI-1*-specific shRNAs (Fig 2B). In contrast to a previous report,[12] similar findings were made for bortezomib, which was confirmed in an independent dataset (Figs S4). Surprisingly, we also did not observe any long-term impact on the growth of *BMI-1*-silenced MM cells. We successfully generated 19 out of 20 BMI-1 shRNA transduced cell line variants 21 days after transduction (exception: RPMI8226, shRNA #20155). Although there was a minor delay on the outgrowth of *BMI-1* silenced compared to control cell lines immediately after transduction (not shown), we observed no major difference in the growth characteristics of stable selected control or *BMI-1*-silenced cell lines (Fig 2C). Findings from colony formation assays supported these results. A significant impact (73% reduction in colony numbers, $P = 0{\cdot}006$) was only noted with one of two BMI1-shRNAs in RPMI8226, but not OPM2 and KMS12BM cells (Fig 2D). This indicates a non-essential role of *BMI-1* for MM cell growth and survival.

To confirm this unexpected finding we analysed publically available Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 data.[20,21] Although BMI-1 expression was higher in MM cell lines compared to all other tumour cell line models (Figs S5), *BMI-1* was clearly indicated as non-essential gene in 18/18 MM cell lines (median dependency score 0·06, range: −0·17 to 0·33). On the contrary, dependency scores for *IRF4* and *MYC* were <−1 underlining their established role as key MM genes (Fig 2E). This suggests that complete loss of *BMI-1* is similarly ineffective to impair MM cell survival and that BMI-1 is likely not a key molecule for BMI-1 modulator activity.

BMI-1 modulators induce mitotic arrest that is accompanied by loss of myeloid cell leukaemia-1 (MCL-1) and subsequent induction of apoptosis in myeloma cells

Based on the recent disclosure of PTC596 as microtubule inhibitor,[16] we studied the effect of PTC-028 and PTC596 on the MM cell cycle to clarify their mode of action. A significant accumulation of Cyclin B1 protein levels indicated mitotic arrest (Fig 3A), which was further confirmed via May-Giemsa Grünwald stainings, cell cycle analysis, as well as assessment of phospho-Serine 28 levels of Histone H3 via flow cytometry (Fig 3B–D). The induction of mitotic arrest

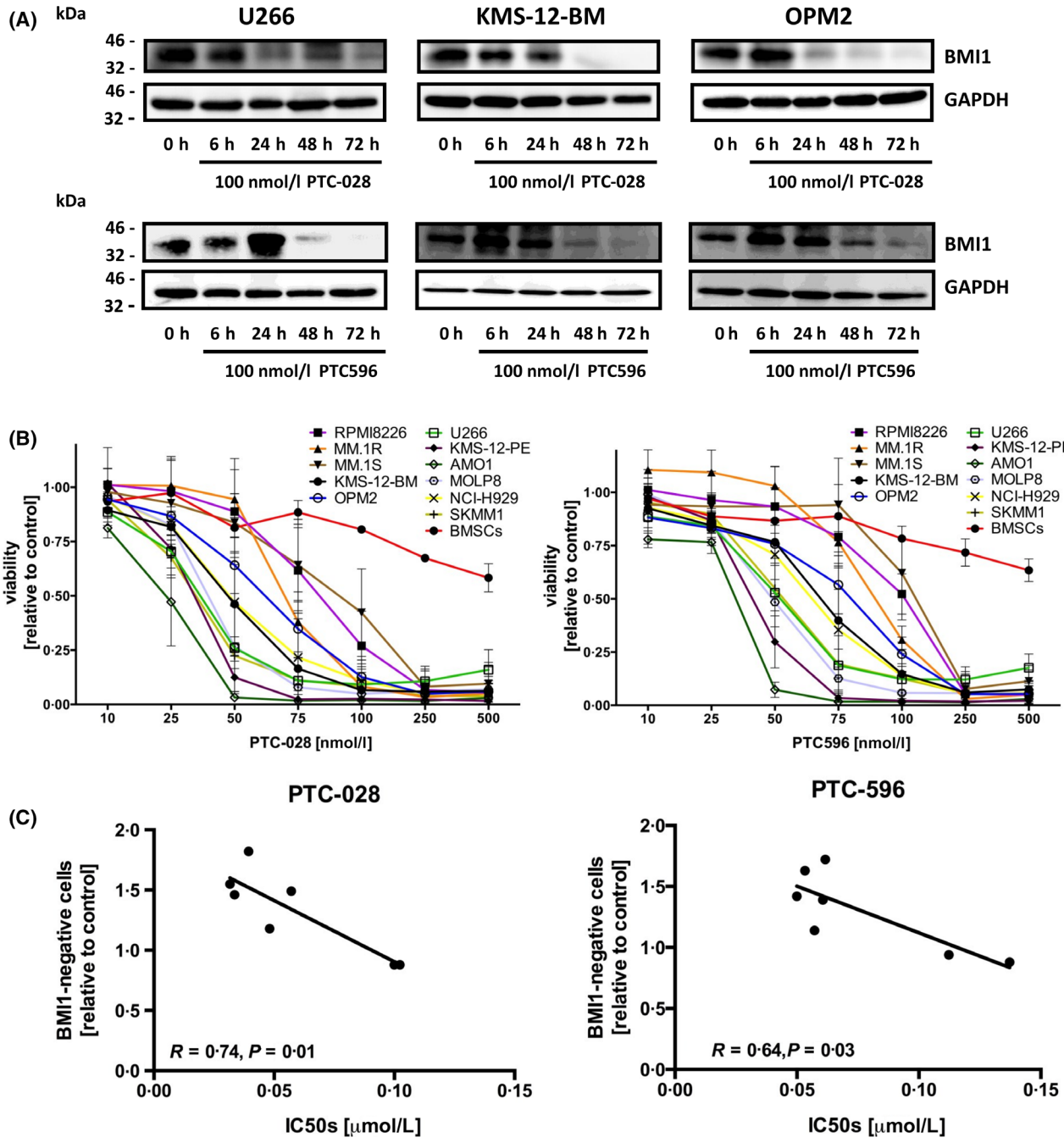

Fig 1. BMI-1 modulators target BMI-1 and myeloma cell viability. (A) BMI-1 protein levels after PTC-028 and PTC596 treatment. (B) Viability curves 96 h after PTC-028 and PTC596 treatment in a panel of human MM cell lines and BM stromal cells (BMSCs). Graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates. (C) Intracellular determination of BMI-1 protein levels via flow cytometry. Linear regression analysis demonstrated a significant association between the accumulation of BMI-1 negative cells (relative to untreated control cells) 24 h after treatment and PTC-028 or PTC596 IC_{50} levels. Y-axis indicates the relative increase of BMI-1 negative cells in treated compared to untreated MM cells ($\frac{BMI\ 1\ negative\ cells\ post\ treatment}{BMI1\ negative\ cells\ without\ drug\ exposure}$). Corresponding Pearson correlation coefficients and *P* values are shown in the graphs.

was in concert with upregulation of mitotic genes at the mRNA level [e.g. Cyclin B1 (*CCNB1*), aurora kinase A (*AURKA*), baculoviral IAP repeat containing 5 (*BIRC5*)]. In addition, although not reaching statistical significance, *MCL-1* mRNA expression was downregulated suggesting loss of MCL-1 (and thus reduced viability) in response to mitotic arrest (Table S4).

Serial assessment of MM cell lines KMS-12-BM and U266 after PTC-028 treatment clearly showed that the induction of apoptosis is preceded by G2/M cell cycle arrest (Fig 4A, B).

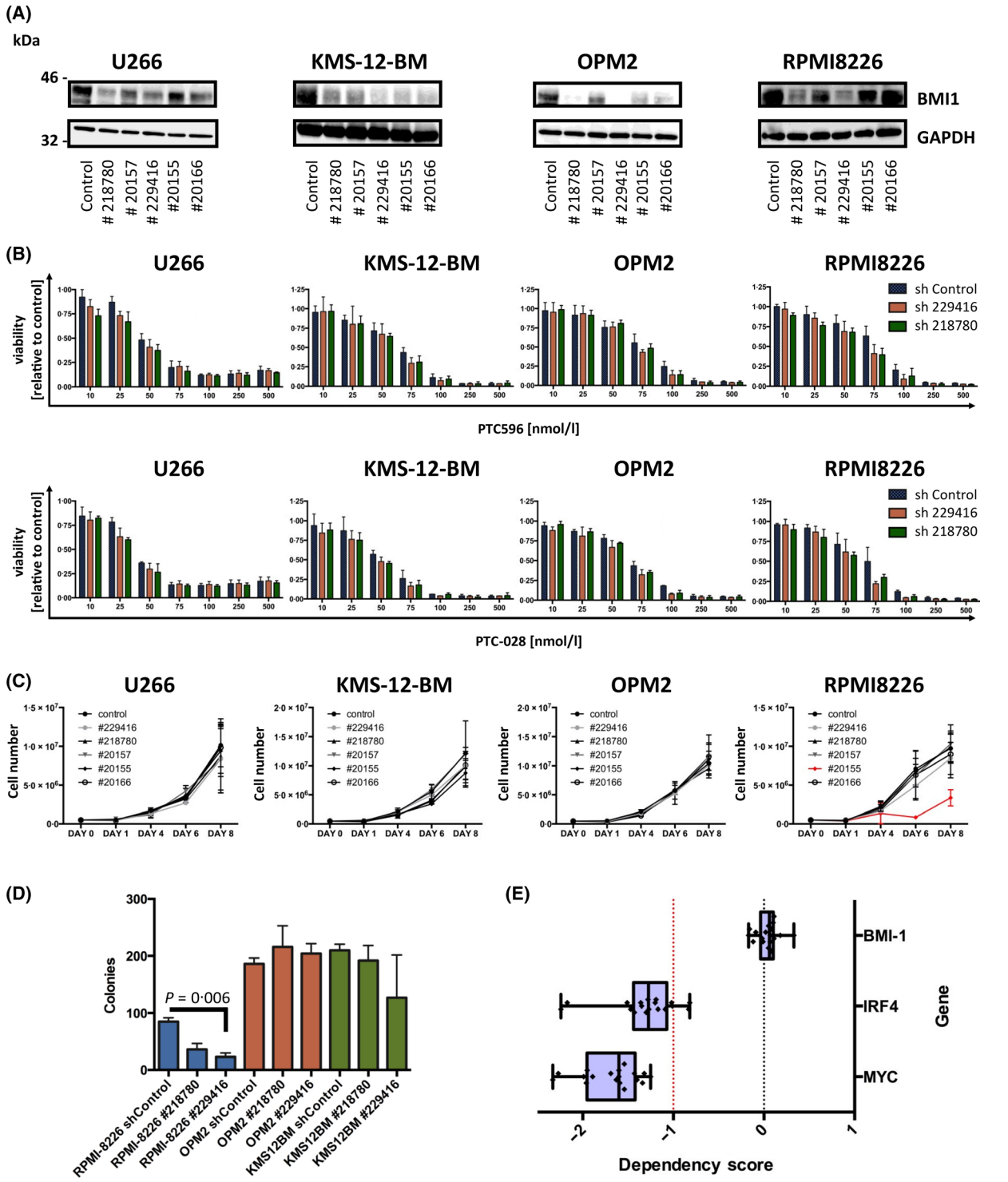

Fig 2. BMI-1 is dispensable for the activity of BMI-1 modulators and myeloma cell growth. (A) Protein expression of stable selected cell line variants transduced with five distinct shRNAs targeting BMI-1 in four MM cell lines. (B) PTC596 and PTC-028 are similar effective in *BMI-1*-silenced and control (scrambled shRNA) MM cell line variants. Viability assessment was performed 96 h after BMI-1 modulator treatment. (C) No difference was observed between the cell growth of control (scrambled shRNA) and *BMI-1*-silenced MM cell line variants. Cell numbers were determined at the indicated time points using a coulter counter. Graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates. (D) Colony formation assays with three MM cell lines transduced with either control or BMI-1-specific shRNAs. (E) CRISPR data demonstrate that BMI-1 is a non-essential gene in MM. IRF4 and MYC served as control (dependency score of zero indicates non-essential genes, Dependency score of −1 indicates common essential tumour genes). The graph displays median dependency scores ± interquartile range of 18 MM cell lines.

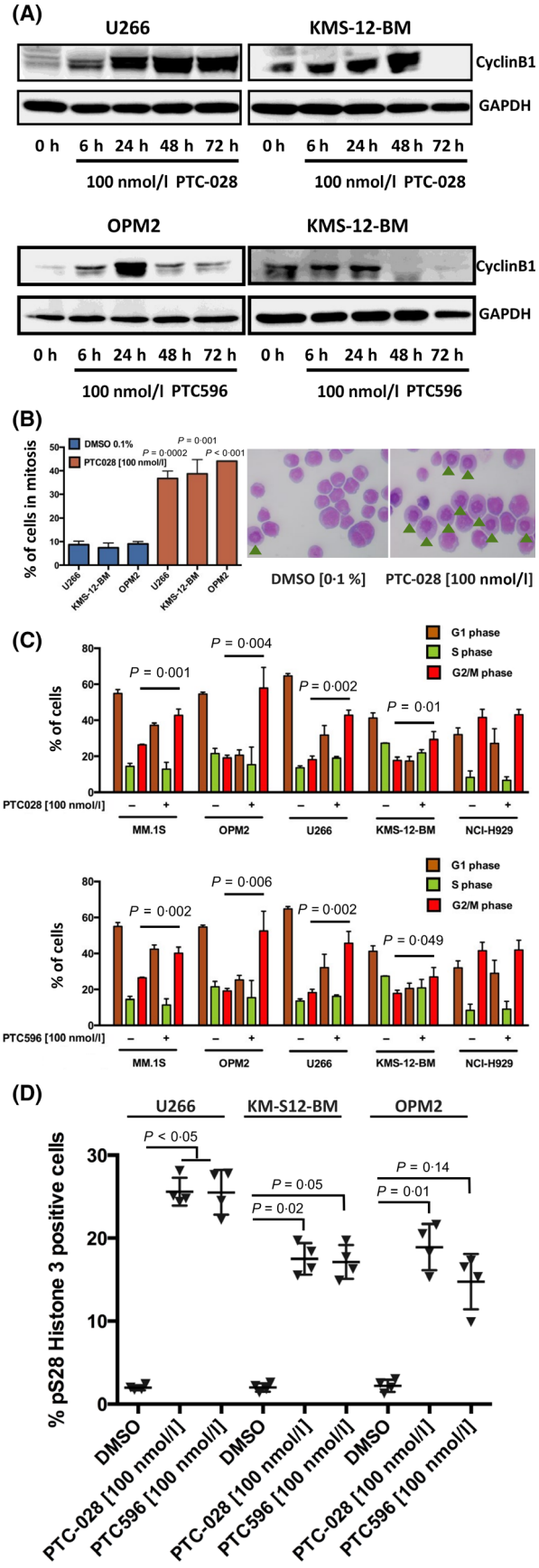

Fig 3. BMI-1 modulators induce a potent mitotic arrest in myeloma cells. (A) Cyclin B1 protein levels after PTC-028 and PTC596 treatment. (B) Mitotic arrest assessed by May-Giemsa Grünwald staining. Representative images (×60 magnification) of KMS-12-BM cells 24 h after treatment with PTC-028 are shown (green arrowheads indicate mitotic cells). (C) Cell cycle analysis via flow cytometry indicated BMI-1 modulator induced accumulation of MM cells in mitosis 24 h after treatment (brown: G1 phase, green: S phase, and red: G2/M phase). (D) Histone H3 phospho-Serine 28 levels were assessed by flow cytometry to further confirm the induction of mitotic arrest 24 h after treatment with either PTC-028 or PTC596 at 100 nM, respectively. Graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates (n = 4 four biological replicates in case of pS28 H3 evaluation). P values indicate significance in comparison with untreated (0·1% dimethyl sulphoxide [DMSO]) cells.

The decrease in MCL-1 protein levels was coupled to this process and linked to the presence of cleaved caspase 8 and 9 at 6–48 h after treatment (Fig 4C, D). In accordance with the reported proteasomal degradation of MCL-1 during prolonged mitosis,[22] we found that BMI-1 modulator induced loss of MCL-1 depends on proteasome activity (Fig 4E). Dose-dependent induction of apoptosis was subsequently confirmed in additional MM cell lines and shown to be preceded by the presence of cleaved PARP and loss of the mitochondrial membrane potential (Fig 5A–C). Importantly, BMI-1 modulators were active in colony formation assays, in the presence of stromal support, as well as in primary MM cells (Fig 5D–F).

BMI-1 modulators affect MYC and AKT signalling activity

BMI-1 was initially reported to promote MYC-driven lymphomagenesis.[3] Hence, we explored whether BMI-1 modulators interfere with the activity of MYC and indeed observed reduced MYC protein expression in response to PTC-028 and PTC596 (Fig 6A). This was corroborated by MYC activity assays showing significantly reduced DNA binding activity 24 h after treatment (Fig 6B). In addition, we observed loss of Forkhead box protein M1 (FOXM1) protein levels (Fig S6), another factor implicated in high-risk MM,[23] as well as reduced AKT signalling activity due to a loss of total AKT1 levels (Fig 6C). No impact was found on extracellular signal-regulated kinase (ERK) or glycogen synthase kinase-3 beta (GSK3b) signalling activity (not shown).

BMI-1 modulation in combination with BH3 mimetics or enhancer of zeste homologue 2 (EZH2) inhibition is synergistic

To define combination partners for BMI-1 modulators in the clinical setting, we tested a diverse set of approved and experimental anti-MM agents. Among approved anti-MM drugs [proteasome inhibitors, immunomodulatory imide

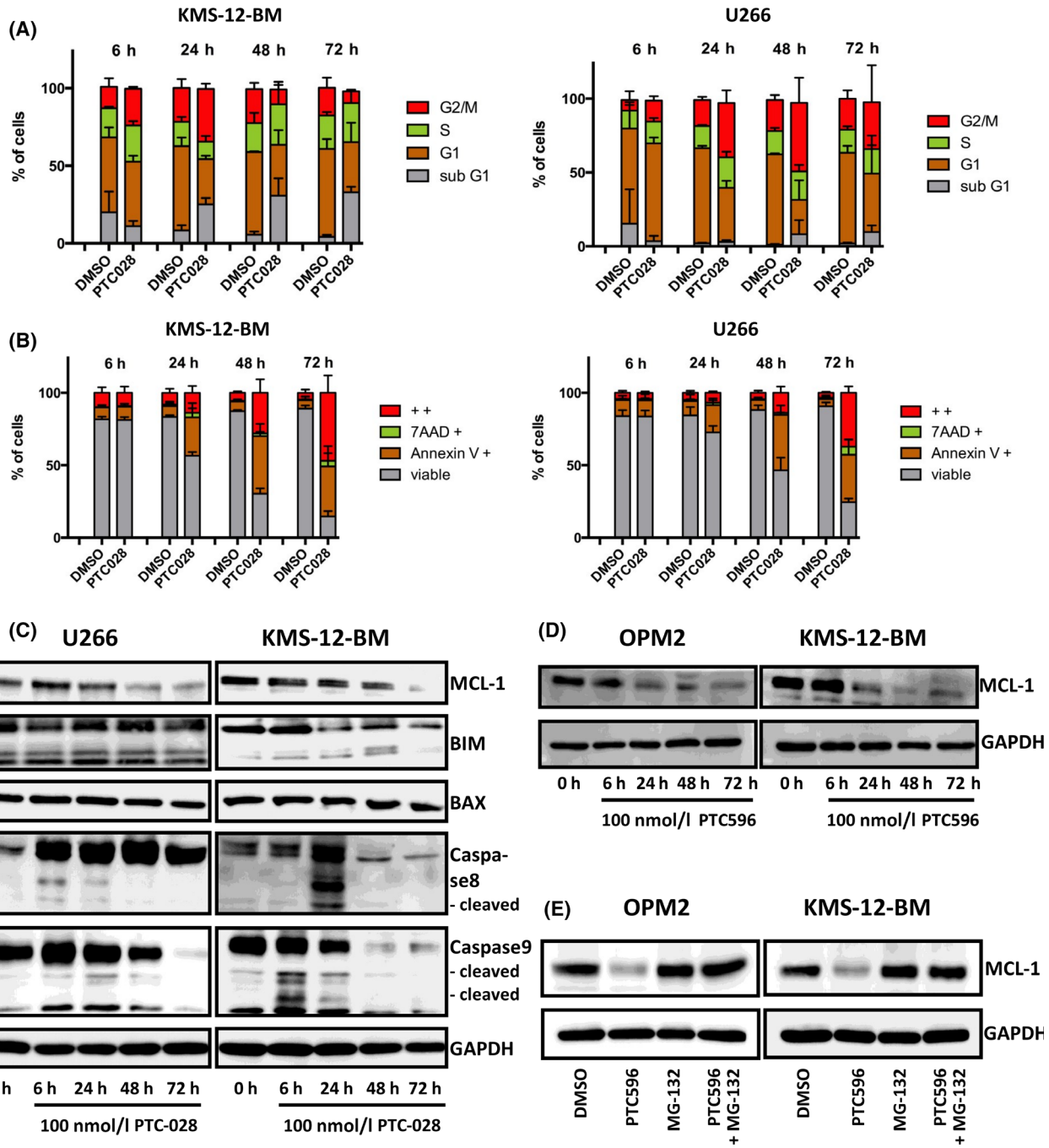

Fig 4. BMI-1 modulators induce proteasome-dependent downregulation of MCL-1 and apoptosis in MM cells. (A) Cell cycle and (B) apoptosis time course analyses demonstrate induction of G2/M arrest followed by induction of apoptosis upon treatment with PTC-028. Graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates. ++ indicates double positive (Annexin V and 7-AAD positive) cells. (C, D) The timing of MCL-1 protein level reduction is associated with the induction of apoptosis. (E) MCL-1 protein levels after treatment with PTC596 (18 h, 200 nM) in the presence or absence of the proteasome inhibitor MG-132 (10 μM, 2 h pre-treatment before addition of PTC596).

drugs (IMiDs), dexamethasone, melphalan], synergism was only observed in combination with IMiDs and dexamethasone in BMI-1 modulator less sensitive cell lines (MM.1S, NCI-H929), as well as additive effects in combination with melphalan (Figs S7 and S8).

Combination studies of PTC596 with BH3 mimetics (venetoclax, navitoclax, S63845, A-1331852) and epigenetic modulators (panobinostat, GSK343, SGC-CBP30, JQ1) revealed strong synergism with the Bcl-extra large (Bcl-xL) inhibitor A-1331852 (23 of 27 tested combinations additive

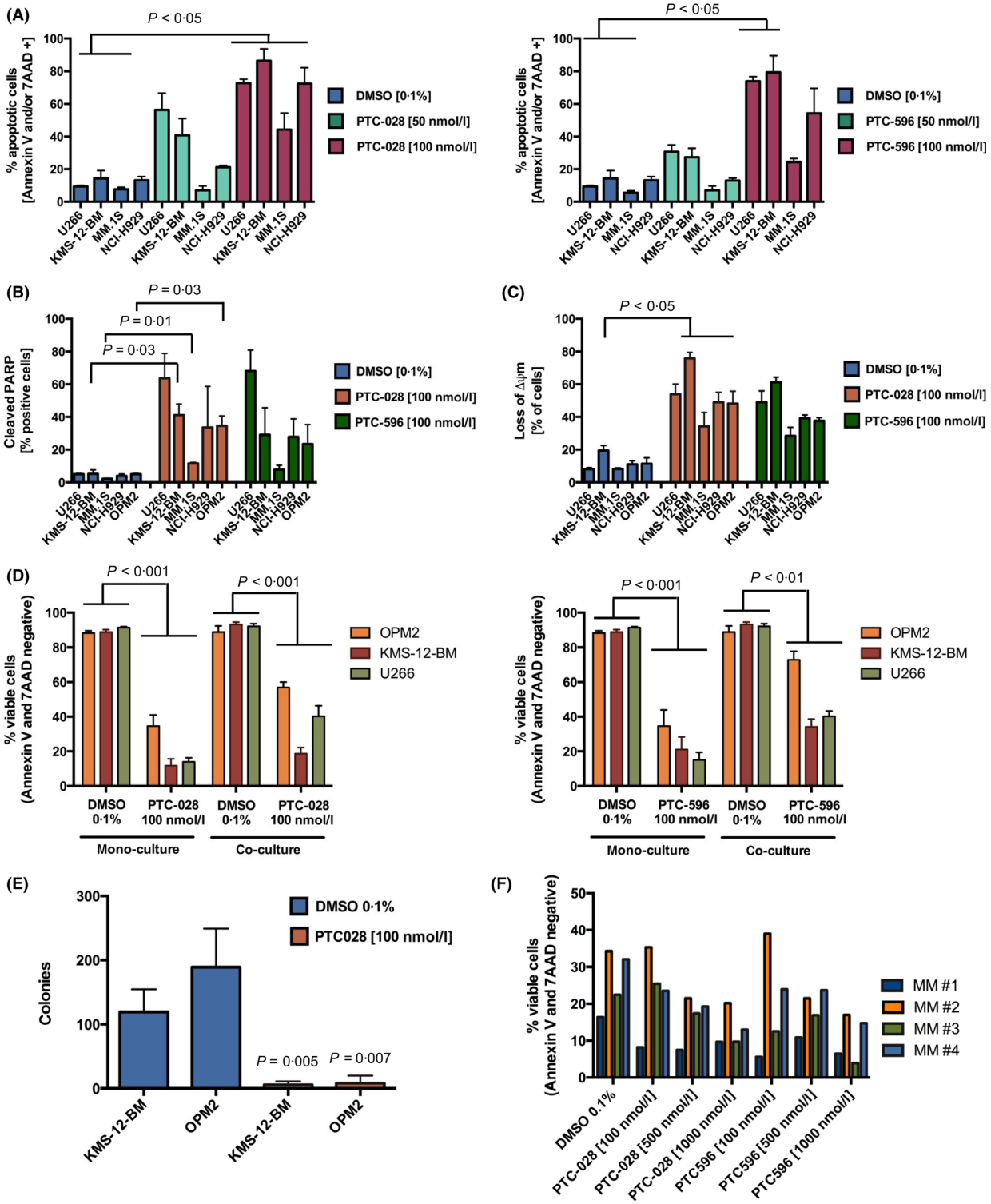

Fig 5. Dose-dependent induction of apoptosis is linked to mitochondrial membrane depolarisation and persists in the presence of stromal support and colony formation assays. (A) Dose-dependent induction of apoptosis (Annexin V/7-AAD staining) in a panel of MM cell lines 72 h after treatment. (B) Detection of cleaved PARP and (C) loss of the mitochondrial membrane potential 48 h after BMI-1 modulator treatment, respectively. (D) BMI-1 modulators induce apoptosis in MM cells in the presence of stromal support (BMSCs) 72 h after treatment. (E) PTC-028 impairs clonogenic replication of MM cells in colony formation assays. (F) The efficacy of PTC-028 and PTC596 was assessed in primary MM cells in the presence of BMSC TERT^{+} cells. Viable cells were determined 72 h after treatment initiation. Experiments with primary cells were repeated once. All other graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates. *P* values indicate significance in comparison with untreated (0·1% dimethyl sulphoxide [DMSO]) cells.

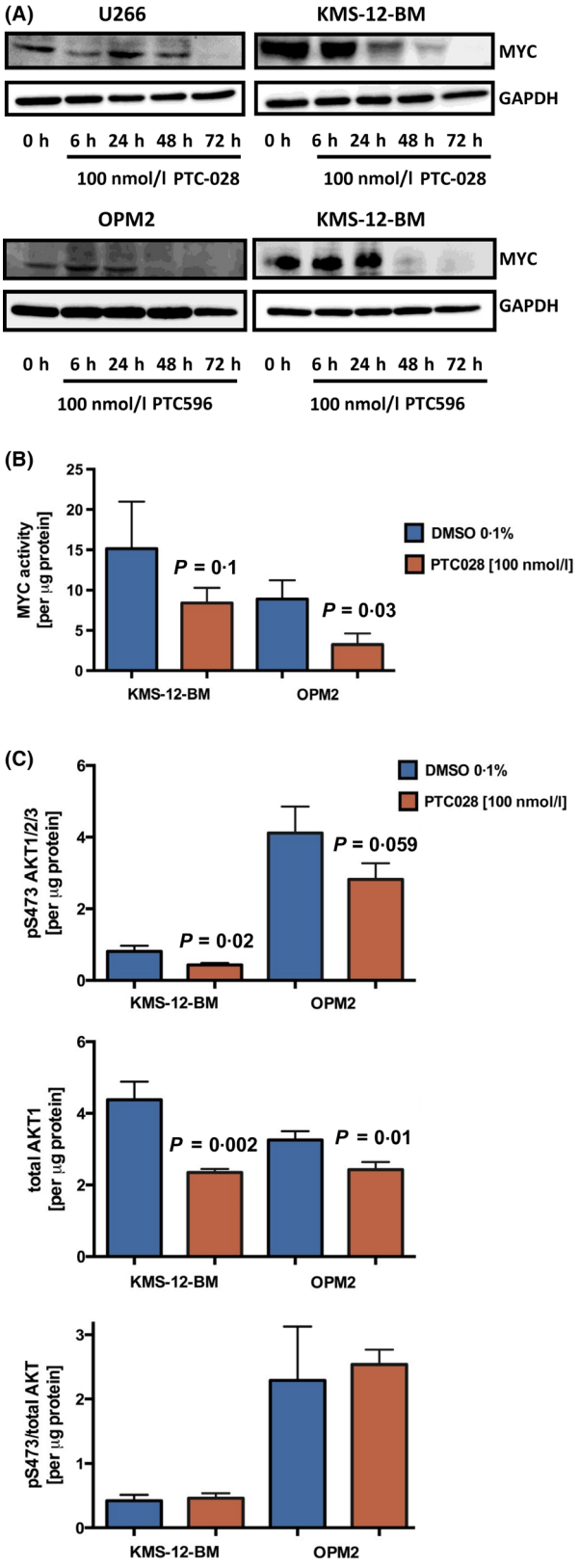

Fig 6. BMI-1 modulators impair MYC and AKT signalling activity. (A) MYC protein levels after PTC-028 and PTC596 treatment. (B) MYC DNA binding activity 24 h after PTC-028 treatment. (C) Phospho-Serine 473, total and phospho/total AKT activity 24 h after treatment with PTC-028. Graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates. *P* values indicate significance in comparison with untreated (0·1% DMSO) cells.

or synergistic) and the EZH2 inhibitor GSK343 in OPM2 and KMS12BM cells at high-dose levels. Panobinostat tended to enhance the efficacy of PTC596 when both drugs were used at the highest dose (Figs S9). In addition, we observed additive activity in combination with the MCL-1 inhibitor S63845 in KMS-12-BM cells (median CI: 0·86, range: 0·31–1·76), as well as synergistic and additive activity with the Bcl-2 inhibitor venetoclax in OPM2 and KMS12BM cells, respectively (OPM2: median CI 0·40, range: 0·21–11·05; KMS-12-BM: 1·11, 0·69–1·50) (Figs S9B-S10A). No synergistic effect was noted with JQ1 and CBP30 (Figs S10B).

To strengthen these findings, we analysed the induction of apoptosis in HMCLs treated with PTC596 ± GSK343 (EZH2 inhibitor), A-1331852 (Bcl-xL inhibitor) or panobinostat used at sub-optimal concentrations in mono- and co-culture. All three drug combinations were significantly more effective than the corresponding monotherapies and/or the only treatment condition that significantly reduced viable cell numbers compared to control. Importantly, this effect persisted in the co-culture setting (Fig 7).

PTC596 shows potent in vivo *activity*

Finally, we explored the activity of the clinical lead compound PTC596 in the 5TGM.1 model of MM. In accordance with our *in vitro* findings, we observed a dose-dependent reduction of BM MM cell infiltration. Twice weekly administration of PTC596 at 15 mg/kg led to a 48·9% reduction of MM-BM infiltration ($P > 0·05$) and weekly treatment with PTC596 at a dose of 30 mg/kg led to a complete eradication (i.e. <lower limit of detection, LOD) of MM cells ($P = 0·1$). These impressive results were confirmed in a second, independent experiment demonstrating a normalisation of paraprotein levels ($P = 0·03$) and absence of MM cells in the BM with PTC596 at 30 mg/kg/weekly ($P = 0·04$) (Fig 8, Fig S11). Toxicological analysis demonstrated no statistically significant impact on white blood cell counts, neutropenia or anaemia. Similarly, we did not observe any impact of PTC596 on body weight; and the performance score (based on the presence and severity of paraplegia, altered posture and diminished activity) completely normalised in mice treated with 30 mg/kg PTC596 (Fig S12, Table 6).

Discussion

The achievement of long-term disease control has evolved as a major treatment goal in MM. To further increase the fraction of patients with prolonged progression-free and overall survival rates, novel treatment opportunities are needed for late-stage (e.g. penta-refractory patients) and high-risk disease. This is most likely realised by targeting well-established key players of MM pathophysiology. BMI-1 is a prominent example of oncogenic factors against which active inhibitors are not available as yet. We therefore analysed the pre-clinical activity of the first-in-class BMI-1 modulators PTC-028 and PTC596.

Both compounds demonstrated striking *in vitro* activity with significantly reduced IC_{50} levels (> 10-fold reduction) compared to PTC-209 in treatment naïve, as well as proteasome inhibitor-resistant MM cell line models. *In vivo*, we observed complete eradication of MM cells in two independent experiments, with no severe impact on animal health (stable body weight, improved performance score). The association between IC_{50} levels and BMI-1 depletion in MM cells suggests that BMI-1 levels before and shortly after treatment (e.g. in circulating tumour cells) could serve as a predictive marker. However, we have to note that this requires access to clinically validated and standardised reagents (antibodies). BMI-1 modulators induce hyper-phosphorylation of BMI-1[15,24] and only reagents that adequately reflect BMI-1 levels in the presence of post-translational modifications can lead to the definition of clinically useful results.

Mechanistically, BMI-1 modulators induced a potent mitotic arrest, followed by loss of MCL-1 and induction of apoptosis. This is in accordance with previous studies analysing the activity of APC/C^{CDC20} inhibitors in MM or the efficacy of PTC596 in pre-clinical tumour models.[16,24,25] Regarding the role of BMI-1 for the growth of MM, cell growth and colony formation assays showed a significant impact of *BMI-1* silencing in only one cell line (RPMI8226). Intriguingly, the postulation of *BMI-1* as an essential MM gene was mainly based on work with RPMI8226. Re-interpreting this study with the data from our present work in mind highlights the less pronounced activity of BMI-1-shRNAs in KMS-12-BM and LP-1 cells (<30% apoptotic cells after doxycycline-inducible *BMI-1* silencing).[11] Accordingly, we only observed a slight delay in the outgrowth of BMI-1-shRNA *versus* control-shRNA transduced cells immediately post-transduction (not shown), but no impact 14 days post-transduction. This indicates that *BMI-1* silencing might have a minor transient effect, but also exemplifies that the term 'essential gene' should be used with more caution and only for targets that truly result in a complete inhibition of tumour cell growth. This is supported by publically available CRISPR data from 18 MM cell lines clearly demonstrating the non-essential role of *BMI-1* in MM. Similar findings were made in pancreatic ductal adenocarcinoma models.[16] In addition, our present data do not support the reported association between BMI-1 expression and sensitivity to bortezomib.[12] However, we cannot exclude a putative essential role for BMI-1 in certain circumstances/disease states (e.g. tumour initiation, tumour propagating cells, drug resistance, distinct stress situations) or in tumour cells embedded in the BM niche where they are faced with different conditions compared to routine *in vitro* culture (e.g. hypoxia). Hence, the specific role of BMI-1 in MM needs to be clarified in future studies that go beyond the routine *in vitro* culture of bulk tumour cell populations.

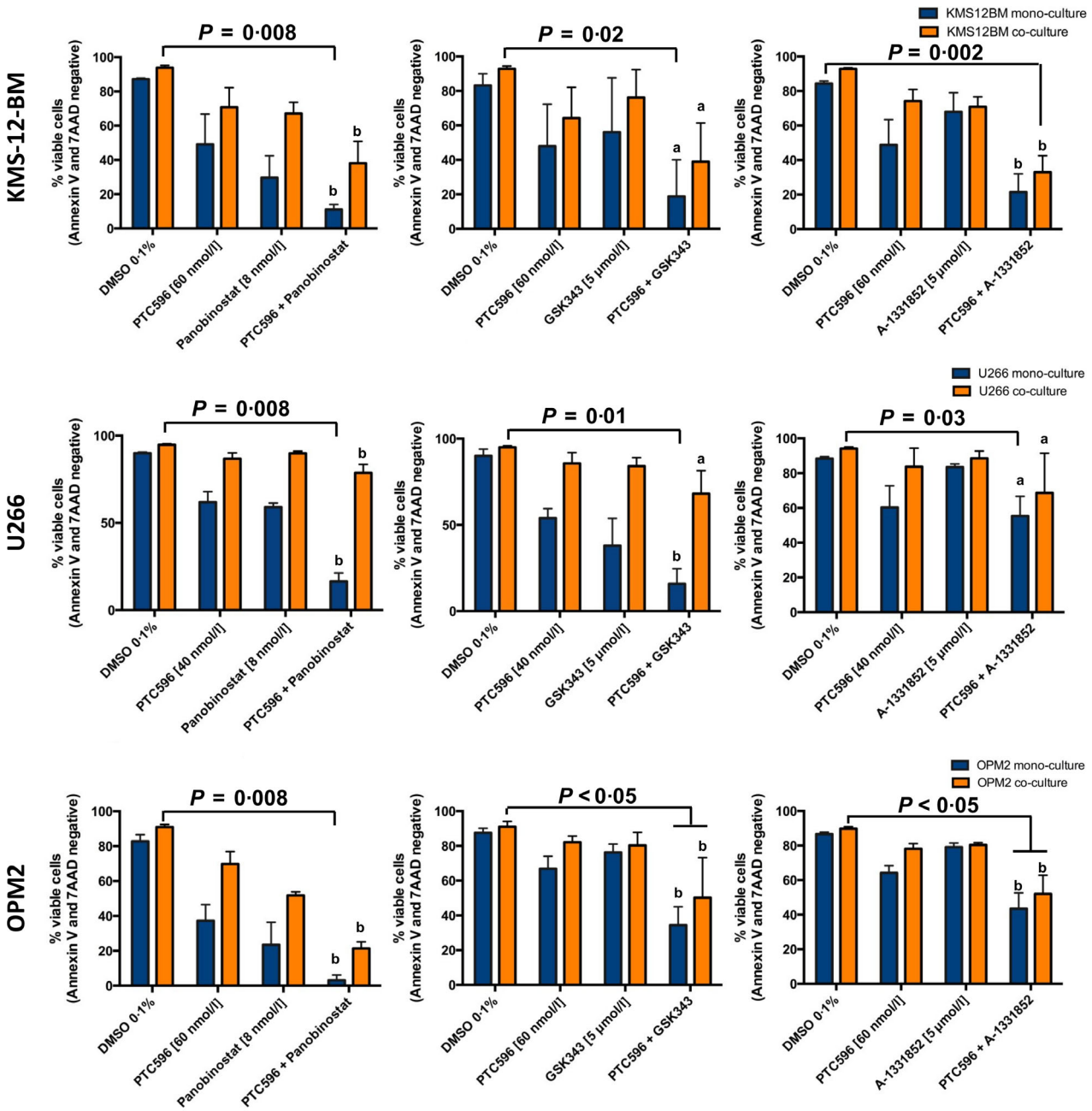

Fig 7. Potentiation of BMI-1 modulator activity with BH3 mimetics and epigenetic compounds. Induction of apoptosis was assessed by Annexin V and 7-AAD staining 72 h after treatment in MM cell mono- and co-culture with BMSCs. All graphs represent the mean ± SD of three independent biological replicates, each with three technical replicates. Multiple comparisons were performed by Kruskal–Wallis and Dunn's multiple comparisons test of pooled results (mono + co-culture, significant differences are indicated by *P* values), as well as within mono- or co-culture experiments (a, $P < 0{\cdot}05$; b, $P < 0{\cdot}01$ vs control cells, i.e. 0·1% dimethyl sulphoxide [DMSO]).

BMI-1 modulators are not direct inhibitors of BMI-1.[15,16] We therefore explored whether BMI-1 is required for the activity of BMI-1 modulators. In line with our observation that *BMI-1* is a non-essential gene, modulation of *BMI-1* expression had no impact on the activity of PTC-028 or PTC596 suggesting that *BMI-1* is not required for drug activity. This is strongly supported by similar results from an independent study in *BMI-1* knockout cells.[16] The non-direct impact of PTC-028 and PTC596 on BMI-1 is also reflected by several *in vitro* findings obtained during the present study that contrast previous reports on *BMI-1* silencing or inhibition.[3,11,14] For instance, here, we noted a G2/M *versus* expected G1 cell cycle arrest or stable *versus* expected upregulation of Dickkopf WNT signalling pathway inhibitor 1

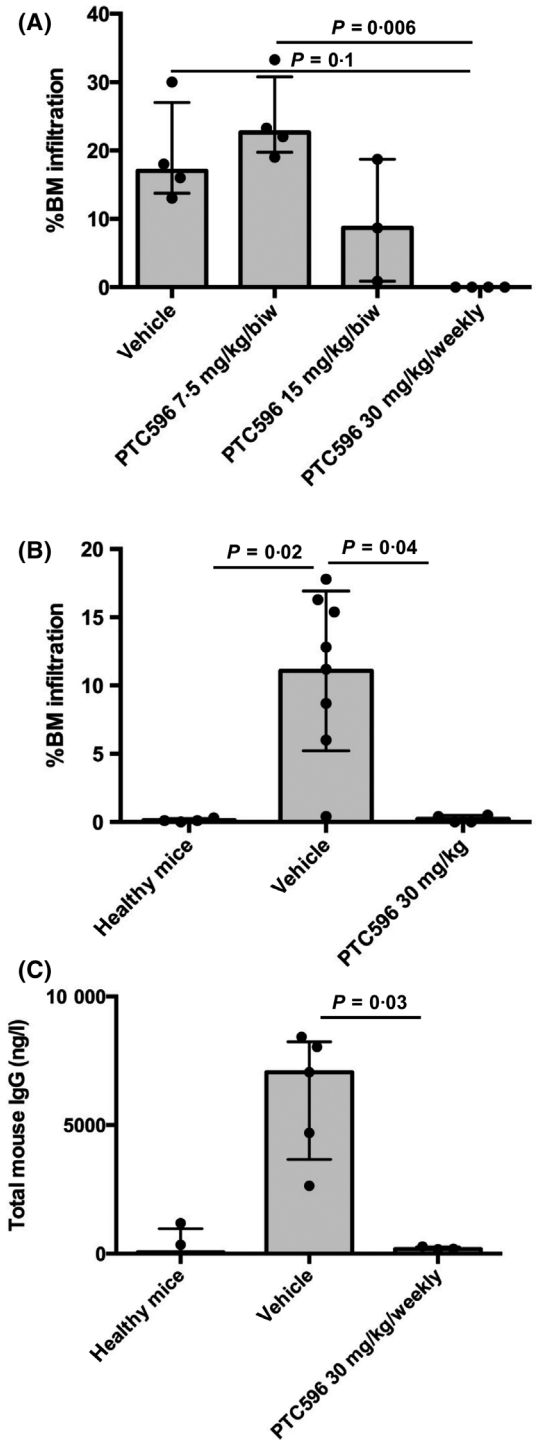

Fig 8. PTC596 halts MM growth *in vivo*. (A) Dose-dependent reduction of bone marrow MM cell infiltration with PTC596. (B) The eradication of MM cells below the lower limit of detection (LOD) was confirmed in a second, independent experiment with PTC596 at 30 mg/kg/weekly. Assessment of bone marrow MM cell infiltration rates and serum paraprotein levels did not indicate the presence of MM. Graphs represent the median ± interquartile range.

(*DKK1*) expression.[14,26] We therefore suggest that the potent anti-mitotic properties of BMI-1 modulators are the basis of their anti-MM activity. Prolonged mitosis results in the rapid loss of MCL-1 protein levels.[22] In parallel, Cyclin B1 protein reduction is significantly delayed. If MCL-1 falls below a critical threshold before the cell enters the next interphase (i.e. before Cyclin B1 falls below a critical threshold) apoptosis is induced.[22,27,28]. Given the essential role of MCL-1 in MM, particularly in late-stage disease and MM cell lines,[29-31] the potent inhibition of microtubule polymerisation leading to metaphase arrest, accumulation of Cyclin B1 and degradation of MCL-1 is likely the primary mechanism of action of BMI-1 modulators in MM.

Drug combination studies revealed synergism with the Bcl-xL inhibitor, A-1331852. BH3 mimetics were previously shown to potentiate apoptosis during mitotic arrest and Bcl-xL is known to compensate for the steady loss of MCL-1 and to sensitise tumour cells to mitotic blockers.[32-34] Thus, BMI-1 modulators most likely prime MM cells for BH3 mimetic treatment due to prolonged mitosis and the associated degradation of MCL-1. Interestingly, we also observed strong synergism in combination with epigenetic drugs (GSK343, panobinostat). Synergism of concurrent BMI-1 and EZH2 blockade was previously described in MM and other entities.[13,35] However, whether this synergism observed in the present study is driven via BMI-1 degradation needs to be clarified in future studies. Moreover, in U266 concurrent EZH2 blockade appeared less effective in co-culture, which might be a consequence of modulation of EZH2 activity by the presence of stromal cells.[36] Histone deacetylase (HDAC) inhibitors were shown to suppress BMI-1,[37] but again, whether this explains the synergism with PTC596 needs to be clarified. A plausible explanation might be the potentiation of mitotic arrest in combination with panobinostat as well as concurrent Bcl-xL inactivation in MM cells, thereby reaching the threshold for inducing death in mitosis in a higher fraction of cells.[38,39]

In conclusion, our present studies demonstrated potent activity of PTC-028 and PTC596 in pre-clinical models of MM, either as a monotherapy or in combination with other experimental therapeutics (most notably Bcl-xL and EZH2 inhibitors). The anti-MM activity of the tested compounds is the result of rapid induction of mitotic arrest and subsequent loss of MCL-1. Nevertheless, indirect modulation of BMI-1 protein levels could serve as predictive biomarker in patients with MM. The findings of the present study are anticipated to lay the foundation for clinical testing of PTC596 in myeloma in the near future.

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Author contributions

Arnold Bolomsky designed the study, performed experiments, analysed the data and wrote the paper. Joséphine Muller, Kathrin Stangelberger, Margaux Lejeune, Elodie Duray, Helene Breid, Christina Pfeiffer and Louise Vrancken designed and performed experiments, as well as analysed the results. Wolfgang Hübl, Martin Willheim, Marla Weetall, Art Branstrom, Niklas Zojer, Jo Caers and Heinz Ludwig participated in the design of experiments. All authors interpreted the data, revised and approved the final version of the manuscript.

Conflicts of interest

This study was supported by a Brian D. Novis Research Grant of the International Myeloma Foundation (IMF) to Arnold Bolomsky. Marla Weetall and Art Branstrom are employees of PTC Therapeutics Inc. All other authors declare no relevant conflicts of interests. PTC-028 and PTC596 were kindly provided by PTC Therapeutics Inc.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table SI. TaqMan assays used for quantitative polymerase chain reaction (qPCR) experiments.

Table SII. Antibodies used for Western Blot experiments.

Table SIII. List of shRNAs used for BMI-1 silencing experiments.

Table SIV. mRNA expression analysis of selected candidate genes in KMS-12-BM cells 6 and 24 hours post PTC-028 or PTC596 treatment.

Table SV. Performance score evaluation during *in vivo* studies. After tumour inoculation, the mice were followed and examined on a daily schedule, evaluating (1) their mobility and movements, (2) their posture and global attitude (3) social behavior and (4) their pilosity as indicated in the table.

Table SVI. Scoring results (performance score) of *in vivo* studies. Treatment with PTC596 was well tolerated by mice that showed no difficulties in their mobility or posture, while the vehicle-treated mice displayed paraplegia or abnormal postures. The table below shows the evolution of the weight and scoring of the treated mice. The only mice that showed clinical signs were the vehicle and low dose (7·5 mg/kg) treated with paraplegia and presence of a hunchback, both related to myeloma development.

Fig S1. IC_{50} levels of PTC596 and PTC-028 are significantly reduced compared to PTC-209. Median IC_{50} levels (based on seven MM cell lines) of PTC596 and PTC-028 are significantly reduced compared to those of PTC-209 ($P < 0{\cdot}05$ compared to median PTC-209 IC50). The graph displays median IC_{50} levels ±interquartile range.

Fig S2. PTC596 and PTC-028 are similar active in models of proteasome inhibitor resistance. The activity of (A) PTC-028 and (B) PTC596 did not differ between proteasome inhibitor sensitive and the corresponding ixazomib or carfilzomib resistant cell line variants. Viability was assessed 96 hours post-treatment. Graphs represent the mean ± SD of three independent experiments performed in triplicates.

Fig S3. BMI-1 overexpression does not affect MM cell growth rates and BMI-1 modulator activity.

Fig S4. BMI-1 expression does not impact on bortezomib sensitivity.

Fig S5. BMI-1 expression in a diverse panel of cancer cell line models.

Fig S6. BMI-1 modulators downregulate FOXM1 protein expression.

Fig S7. Drug combination studies of PTC-028 with established anti-myeloma agents.

Fig S8. Drug combination studies of PTC596 with established anti-myeloma agents.

Fig S9. PTC596 in combination with panobinostat, GSK343, ABT199, and A-1331852.

Fig S10. PTC596 in combination with S63845, navitoclax, CBP30, and JQ1.

Fig S11. Representative flow cytometry images of MM bone marrow infiltration in the 5TGM.1 murine model.

Fig S12. Toxicological and well-being data of *in vivo* experiments. No significant impact of PTC596 treatment was observed on the indicated clinical parameters.

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