Citation: Blood Cancer Journal (2012) 2, e50; doi:10.1038/bcj.2011.44
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www.nature.com/bcj

ORIGINAL ARTICLE

Inhibition of cell cycle progression by dual phosphatidylinositol-3-kinase and mTOR blockade in cyclin D2 positive multiple myeloma bearing IgH translocations

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Multiple myeloma (MM) is a clinically and genetically heterogenous cancer where tumour cells have dysregulated expression of a D-type cyclin, often in association with a recurrent IgH translocation. Patients whose tumour cells express cyclin D2, with the translocation t(4;14) or t(14;16), generally have more proliferative disease and inferior outcomes. The phosphatidylinositol-3-kinase (PI3K) pathway is a major regulator of D-type cyclin expression and cell cycle entry. We evaluated the effect of PI3K pathway blockade on cell cycle behaviour in MM cells, investigating differences between cyclin D2- and cyclin D1-expressing tumours. MM cell lines and primary bone marrow $CD138^+$ MM cells were exposed to the pan-PI3K/mTOR inhibitor, PI-103, and assessed for cell cycle profiles, [^{3}H]-thymidine uptake and cell cycle proteins. We report, in both cell lines and primary MM cells, that PI-103 induced cell cycle arrest with downregulation of cyclin D2 and CDK4/6 in MM cells expressing cyclin D2 via t(4;14) or t(14;16) translocations. Cells expressing cyclin D1 via t(11;14) were insensitive to PI-103, despite exhibiting inhibition of downstream signalling targets. In primary MM cells, PI-103 enhanced the anti-proliferative effects of anti-MM agents. Treatment paradigms including blockade of the PI3K/mTOR pathway should be targeted at patients with IgH translocations associated with cyclin D2 overexpression.

Blood Cancer Journal (2012) **2,** e50; doi:10.1038/bcj.2011.44; published online 13 January 2012

Keywords: multiple myeloma; cyclin D; cell cycle; PI3K/mTOR; PI-103

INTRODUCTION

Multiple myeloma (MM) is the second most common haematopoietic cancer and is caused by an accumulation of malignant plasma cells in the bone marrow. Although the recent use of proteasome inhibitors and IMiDs have improved response rates and overall survival, this cancer remains incurable in the vast majority of patients and new treatments are urgently needed. We now understand that the considerable clinical heterogeneity corresponds to different biological subtypes that are underpinned by distinct genetic changes.[1,2] This poses a great challenge to the development of effective therapies, as responses to novel therapies may vary depending upon the biological subtype of disease. A subgroup of patients have inferior outcomes: poor response rates, shorter response duration and overall survival.[3] Despite some evidence of improved responses to novel agents, a significant unmet need remains for such patients. Importantly, these patients fall into identifiable subgroups based on particular genetic lesions and expression profiles. The translocation/cyclin D classification is based on dysregulation of a D-type cyclin, together with the presence of a recurrent translocation that places an oncogene under the control of the strong IgH enhancer. This classification is validated by distinct expression profiles and by notable differences in clinical features and outcome.[1,4,5] Patients whose tumour cells carry the t(4;14) involving the FGFR3/MMSET genes and the t(14;16) or t(14;20) involving the maf oncogene, fall into subgroups with inferior outcomes.[3] This subtype of disease has a more proliferative profile and is associated with dysregulated expression of cyclin D2, which is the major D-type cyclin expressed by B-lineage cells, regulating cell cycle entry in B-cell development and activation. Unlike normal plasma cells that have irreversibly exited the cell cycle, MM cells retain the capacity for self-renewal, and higher proliferative rates signify a worse outcome.[6] Thus, anti-proliferative therapies aimed at disrupting the cellular networks that control cell cycle entry may hold the key to more effective treatments in these patient subgroups.

Until recently, however, the mechanisms responsible for mediating cell cycle entry in primary MM cells were not known. We recently showed, for the first time, that MM cells from patients with t(4;14) and t(14;16) respond to mitogenic growth factors with upregulation of cyclin D2, CDK4 and 6, leading to retinoblastoma protein (pRb) phosphorylation and cell cycle entry/progression, thus providing functional validation for the observed D-type cyclin dysregulation in this disease.[7,8] In contrast, MM cells with t(11;14) disease characterized by overexpression of cyclin D1 were less dependent on exogenous stimuli for cell cycle progression.

The phosphatidylinositol-3-kinase (PI3K) pathway is an important target in cancer, as it activates the Akt/Protein kinase B signalling cascade that phosphorylates a number of target molecules to regulate cell growth, proliferation and survival.[9] This pathway mediates the effects of major MM growth factors, such as insulin-like growth factor-I (IGF-I) and interleukin-6 (IL-6) that stimulate proliferation, survival and drug resistance.[10] The PI3K pathway is essential for activation of D-type cyclins and subsequent cell cycle entry in normal B lymphocytes.[11] There is evidence for activation of the PI3K/Akt pathway in MM. Phosphorylation of Akt has been demonstrated in bone marrow biopsies from MM patients,[12] whereas PI3K signalling is activated in response to agents that promote survival, and/or

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Received 03 June 2011; accepted 16 August 2011

chemoresistance, including IL-6, IGF-1, BAFF (B-cell activating factor)/APRIL (A proliferation-inducing ligand), and adhesion to extracellular matrix.[12] In addition, PI3K signalling may be activated by loss of the negative regulator, PTEN, as evidenced in some MM cell lines, or by Ras mutations, present in about 20% of MM tumours.[13] Although early experience with pan-PI3K inhibitors resulted in toxicity to normal tissues in preclinical models, the recent development of isoform-specific compounds with lower toxicity profiles has led to renewed interest in this pathway as a target for anti-cancer therapies. One of the key targets of the PI3K/Akt pathway is mTOR, and feedback signalling from mTOR inhibition (for example, by rapalogs) can lead to PI3K activation thereby attenuating therapeutic effects[14] providing the rationale for employing dual PI3K/mTOR inhibitors.

The aim of this work was to define the effect of dual inhibition of PI3K/mTOR signalling on cell cycle progression and regulatory proteins in MM and to explore differences between different subgroups. PI-103 is a potent and selective inhibitor of class I PI3Ks and mTOR, that blocks proliferation of several solid tumour cell lines and shows therapeutic activity towards human solid tumour xenografts.[15] We hypothesized that, because of aberrant expression of cyclin D2 and reliance on mitogen-activated pathways, MM cells bearing t(4;14) and t(14;16) would be especially vulnerable to PI-103.

MATERIALS AND METHODS

Cell lines

The KMS11, KMS12BM, KMS21BM, KMS26, KMS27 and KMS28PE cell lines were donated by Dr T Otsuki, (Kawasaki Medical School, Okayama, Japan) and MM1-S by Dr S Rosen (Northwestern University, Chicago, IL, USA). Other myeloma cell lines (OPM2, NCI-H929, U266, JIM1 and LP1) were obtained from ATCC. Cells were passaged in RPMI-1640 containing 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 10% foetal calf serum (FCS). For IGF-I stimulation experiments, cells were washed in phosphate-buffered saline and seeded at 5×10^5 cells/ml in RPMI-1640 containing 2 mM L-glutamine and 100 units/ml penicillin/streptomycin, with or without 10% FCS or 100 ng/ml rhIGF-I (R&D Systems, Abingdon, Oxford, UK). PI-103-HCl (Tocris Bioscience, Bristol, UK) was prepared in 1 mM DMSO and diluted to a final concentration of 0.125–2 μM in culture medium. Control cultures were treated with an equivalent amount of DMSO.

Primary samples

BM aspirates and peripheral blood samples were obtained from MM patients after informed consent and with full ethical approval from an institutional review board. $CD138^+$ plasma cells were isolated from BM aspirates or peripheral blood using MACS CD138 MicroBeads (Miltenyi Biotec, Bisley, Surrey, UK); purity of malignant cells was >90%. Samples were cultured as described for human multiple myeloma cell lines (HMCL) or snap frozen and stored at -80 °C before analysis by western blotting.

Cell proliferation/viability analysis

Cell viability following PI-103 treatment of HMCL was performed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQueous Assay; Promega, Southampton, UK). Cells were cultured in triplicate at 10^5 cells/well and analysed according to the manufacturer's instructions.

Cell proliferation was monitored by [^{3}H]-thymidine incorporation. Cells were cultured in triplicate at 10^5 cells/well, and 1 μCi of [^{3}H]-thymidine was added for the final 4 h of the indicated culture period. Cells were harvested using an automated cell harvester and [^{3}H]-thymidine incorporation into DNA was quantified using a scintillant sheet and counter (Meltilex; Wallac, Turku, Finland). Data presented are mean counts per minute ± s.d.

Flow cytometric analysis

Cell cycle analyses were performed by propidium iodide staining for DNA content and flow cytometric analysis. Briefly, cells were fixed in 90% ethanol/10% phosphate-buffered saline before incubation with 50 μg/ml DNase-free RNase and 50 μg/ml propidium iodide in staining buffer (0.1% sodium citrate, 0.1% Triton X-100) for 30 min at 37 °C, and analysed by flow cytometry (Cyan ADP; Dako, Ely, Cambridgeshire, UK).

Western blotting

Cells were incubated in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 'Complete' protease inhibitors (Roche, Burgess Hill, UK) and 'Phosphatase inhibitor cocktail II' (Merck Chemicals Ltd, Beeston, Nottingham, UK)) on ice for 15 min. In all, 20 μg of lysate was separated by SDS-PAGE and transferred to nitrocellulose. Antibodies to cyclin D1 (DCS-6), cyclin D2 (M-20), CDK4 (C-22), CDK6 (C-21), pRb (C-15) and $p27^{Kip1}$ (C-19) were purchased from Santa Cruz Biotechnology (Insight, Wembley, UK); phospho-pRB ($Ser^{807/811}$) from New England Biolabs, Hitchin, UK; phospho-PKB (Ser^{473}) from Invitrogen, Paisley, UK; Foxo 1, PKB, GAPDH, phospho-Foxo 1/4 and phospho-Foxo3a from Cell Signalling Technologies, Danvers, MA, USA; Foxo3a from Abcam, Cambridge, UK and actin (Ab-5) from BD Transduction Laboratories, Oxford, UK.

Blockade of PI3K/mTOR *in vivo*

All procedures involving the use and care of animals were performed in accordance with the Animal Scientific Procedures Act (1986) and licensed by the Home Office. β2microglobulin null/NOD/SCID animals were housed in specific pathogen-free conditions in individually ventilated cages (Biozone, London, UK and Tecniplast, Buguggiate, Italy) and supplied with sterile food, water and bedding. A total of 10^7 myeloma cells were subcutaneously injected into the left flank of 8-week-old unconditioned animals. Animals were randomly assigned into control and treatment groups. The dual PI3K/mTOR inhibitor NVP-BEZ235 (LC Laboratories, Woburn, MA, USA)[16] was administered intraperitoneally daily at 40 mg/kg beginning 1 week after subcutaneous injection of myeloma cells. At the end of study, tumours were excised, weighed and about 30 mg were snap-frozen and homogenized in lysis buffer. Total protein extracts were kept at −20 °C until being processed for immunoblotting.

Statistics

Differences between conditions were analysed using paired or unpaired two-tailed Student's *t*-test, and are designated as means ± s.d. or s.e.m., as appropriate. Differences were considered significant for $P < 0.05$.

RESULTS

Effects of PI3K/mTOR pathway Inhibition by PI-103 on growth of myeloma cell lines depends on IgH translocation status

To determine the efficacy of PI-103 in blocking MM cell proliferation, we initially performed cell viability assays (MTS) in selected HMCL with different underlying IgH translocations. Figure 1a shows a dose-dependent decrease in the number of viable MM1S and NCI-H929 cells cultured in the presence of PI-103, with IC_{50} values of approximately 0.5 and 0.25 μM, respectively. In contrast, KMS12-BM cells were less sensitive, as an IC_{50} was not achieved with up to 2 μM. NCI-H929 cells harbour the t(4;14) IgH translocation that indirectly upregulates cyclin D2 mRNA, while MM1S cells carry a t(14;16) that upregulates cyclin D2 via the c-MAF transcription factor. KMS12-BM, on the other hand, expresses cyclin D1 constitutively via t(11;14).

These data suggest that the response to PI3K blockade in MM tumours may segregate with IgH translocation/D-type cyclin group. To determine if this was more widely applicable, we performed MTS assays on a panel of HMCL representing the three major IgH translocation groups in MM (Figure 1b). We performed these assays in both FCS and IGF-I, which is a major PI3K-dependent, physiological mitogenic growth factor for MM. These data confirmed that t(11;14)-bearing MM cells are less sensitive to PI-103 than those carrying a t(14;16) or t(4;14).

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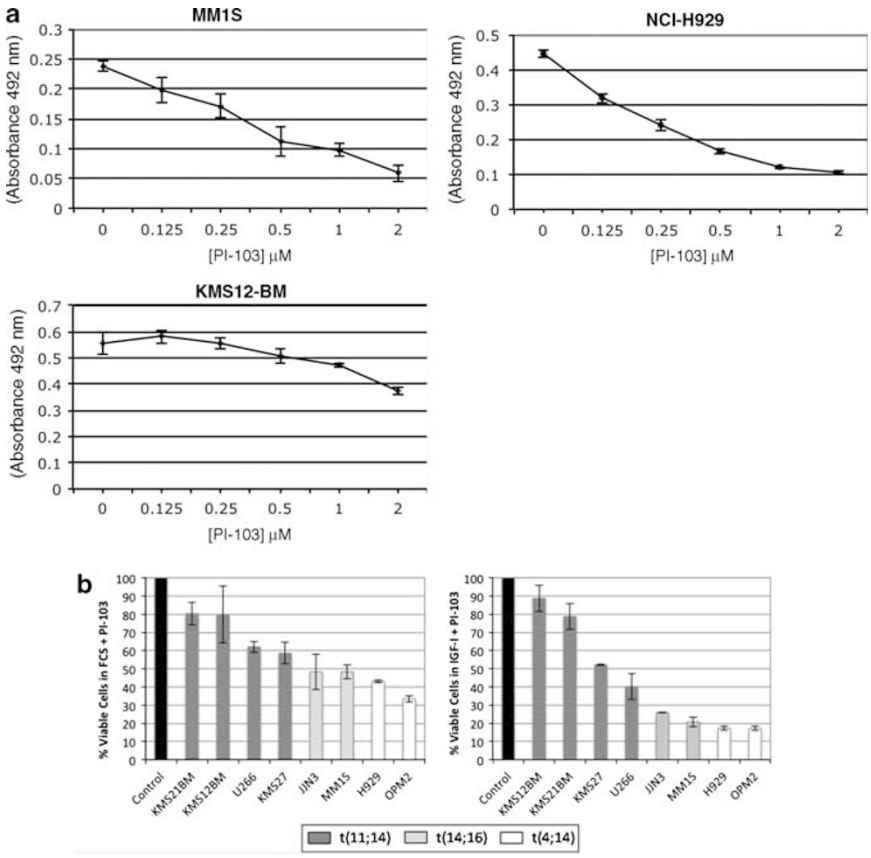

Figure 1. Activity of PI-103 on growth of HMCL. (**a**) MM1S, NCI-H929 and KMS12-BM lines were treated with increasing concentrations of PI-103 in standard growth medium (RPMI-1640 and 10% FCS). The relative number of viable cells was estimated by MTS assay at 24 h (mean ± s.d. of triplicates in one representative experiment). (**b**) MTS viability assays were performed on a panel of HMCL treated with 1 μM PI-103 for 24 h in the presence of 10% FCS (left panel) or 100 ng/ml IGF-I (right panel). The key indicates the IgH translocation type for each HMCL. Data shown are means ± s.d. of triplicates in one representative experiment.

Furthermore, PI-103-mediated growth inhibition was observed when added to MM cells cultured in either FCS or IGF-I.

Cell cycle effects of dual PI3K/mTOR pathway inhibition in myeloma cell lines is dependent on D-type cyclin/translocation status

These data clearly demonstrated that PI-103 treatment led to a reduction in cell number, and because this inhibitor has been reported to act primarily by cytostatic rather than cytotoxic mechanisms in other tumour types,[17] we investigated effects on the cell cycle.

We started with a detailed analysis of both the PI-103-sensitive cyclin D2-expressing MM1S line and the PI-103-resistant, cyclin D1-expressing KMS12-BM line. Figure 2 shows the results of one representative experiment in which cells were treated with PI-103 in the presence or absence of FCS, IGF-I or IL-6, another PI3K-dependent MM growth factor. Both IGF-I and IL-6 increased the proportion of MM1S cells entering the cell cycle (Figure 2a, upper panel). After 24 h, 43% of cells stimulated with IGF-I were in S and G2/M phases, compared with 27% with IL-6, 21% with FCS and 11% in serum-free medium. PI-103 inhibited cell cycle progression in all cases to the level of the serum-free control. Representative cell cycle plots are shown in Figure 2b (upper panel). The increased cell cycle entry observed with all three mitogenic stimuli was accompanied by an upregulation of cyclin D2 as well as CDK4 and 6, resulting in increased phosphorylation of pRb at Ser807/811, a CDK4/6-specific site (Figure 2c, left panel). Total pRb protein was also increased, because phosphorylation of pRb by CDKs releases pRb-mediated transcriptional repression of E2F transcription factors, which induce the expression of genes required for S-phase progression as well as pRb itself. In contrast, expression of the CKI p27 was downregulated by mitogens. Meanwhile, PI-103 treatment inhibited the induction of cyclin–CDK complexes to levels below that of the serum-free control and increased p27 expression, resulting in decreased pRb phosphorylation and cell cycle exit.

In KMS12-BM, however, FCS, IGF-I and IL-6 failed to increase the number of cells in S and G2/M phases compared with serum-free medium (approximately 40% in all cases), and there was no inhibition by PI-103 (Figures 2a and b, bottom panels). Interestingly, IGF-I slightly decreased the number of KMS12-BM cells in S phase, whereas PI-103 reversed this negative effect. In keeping with the lack of effect of growth factors or PI-103 on cell cycle progression, there was very little change in the expression of cyclin D1 and associated cell cycle control proteins with the different treatments (Figure 2c, right panel).

We confirmed these effects of PI-103 on IGF-I-mediated cell cycle entry/progression in a large panel of HMCL with known IgH translocations (Figure 3). HMCL harbouring t(4;14) or t(14;16) were more responsive to IGF-I and more sensitive to inhibition by PI-103 than HMCL with the t(11;14) translocation (Figure 3a). In HMCL harbouring t(4;14) or t(14;16), IGF-I increased the percentage of cells in S and G2/M phases from 24 ± 3% to 31 ± 3% ($P<0.05$) and from 27 ± 7% to 41 ± 3% ($P<0.05$), respectively, whereas in t(11;14)-bearing HMCL, cell cycle progression was unaffected by

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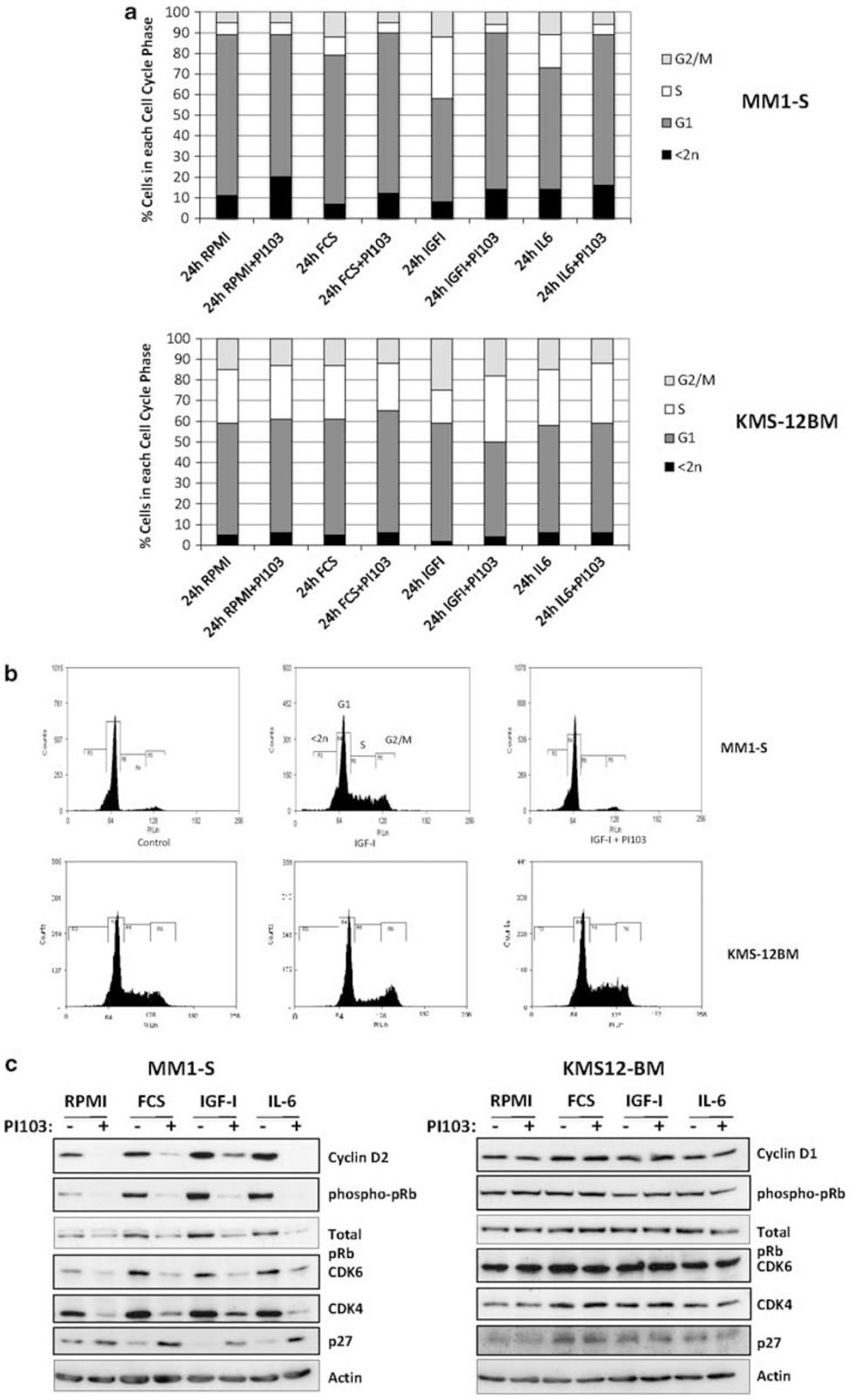

Figure 2. Effect of PI-103 on cell cycle status in MM1S and KMS12-BM. Cells were cultured for 24 h in either serum-free medium (RPMI-1640) or RPMI-1640 plus 10% FCS, 100 ng/ml IL-6 or 100 ng/ml IGF-I ± 1 μM PI-103 and analysed for DNA content by propidium iodide staining and flow cytometry. (**a**) The percentage of cells in each cell cycle phase with the different culture conditions is indicated by the shading within the bars, according to the key. Data shown are from one of three representative experiments. (**b**) Representative cell cycle plots indicating DNA content are shown for MM1S (upper panels) and KMS12-BM (lower panels). (**c**) Western blots for MM1S and KMS12-BM, cultured for 24 h as above, were probed with antibodies against cell cycle control proteins, as indicated. Anti-actin is shown as a loading control.

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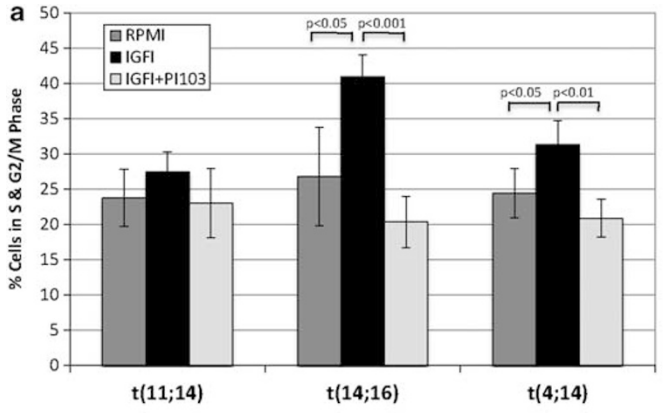

HMCL	D-type Cyclin	IgH Translocation
KMS12BM	D1	t(11;14)
U266	D1, D2	t(11;14)
KMS21BM	D1	t(11;14)
KMS27	D1, D2	t(11;14)
MM1S	D2	t(14;16)
JJN3	D2	t(14;16)
KMS26	D2	t(14;16)
KMS11	D2	t(14;16), t(4;14)
OPM2	D2	t(4;14)
NCI-H929	D1, D2	t(4;14)
KMS28PE	D2	t(4;14)
JIM1	D2	t(4;14)
LP1	D2	t(4;14)

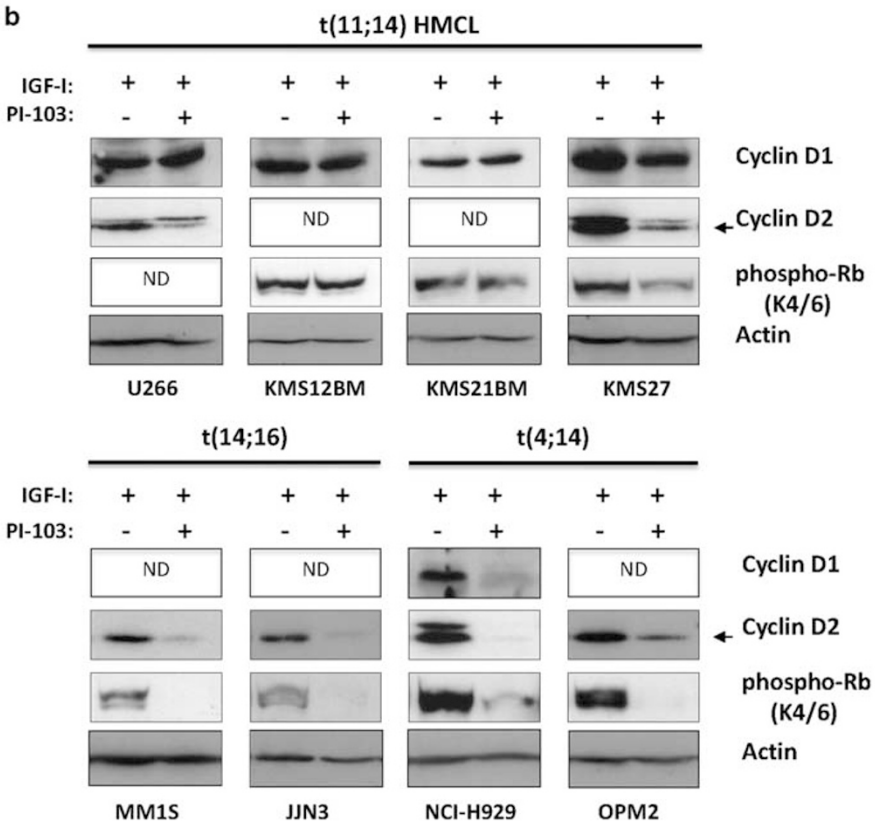

Figure 3. Effect of PI-103 on cell cycle status in a panel of HMCL. Cells were cultured for 24 h in either serum-free medium (RPMI-1640) with or without 100 ng/ml IGF-I ± 1 μM PI-103 before being stained with propidium iodide to assess DNA content. (**a**) Upper panel: The percentage of cycling cells (cells in S, G2 and M phases) for each IgH translocation group with each condition is indicated according to the key. Data are mean ± s.e.m., with $n = 4$ for the t(11;14) group, $n = 4$ for the t(14;16) group and $n = 6$ for the t(4;14) group. Lower panel: Details of the HMCL tested are shown in the table below. (**b**) Representative western blots showing the inhibition of D-type cyclin expression and CDK4/6-specific pRb phosphorylation by PI-103 in HMCL from different IgH translocation groups. Whole-cell lysates were prepared from cells treated for 24 h, as described above. Blots were probed with antibodies against cell cycle control proteins, as indicated. Anti-actin is shown as a loading control. NB: The cyclin D2 antibody cross-reacts with cyclin D1, which appears as a slightly higher molecular weight band. ND, not detected.

IGF-1. Furthermore, PI-103 reduced the number of cycling cells to 21 ± 3% in t(4;14) cells ($P < 0.01$) and to 20 ± 4% in t(14;16) cells ($P < 0.001$), but was without effect on t(11;14) cells. The results of these experiments suggest that the effect of PI-103 on viable MM cell numbers may be mediated primarily by alterations in cell cycle, consistent with other tumour types.[15]

Consistent with the observed effects on the cell cycle, PI-103 almost completely abolished the IGF-I-mediated increase in cyclin D2 and phospho-pRb observed in t(4;14) or t(14;16) HMCL, but had little or no effect on levels of cyclin D1 and phospho-pRb in t(11;14) cells (Figure 3b). It should be noted, that unlike primary MM cells,[7] some of the HMCL express both cyclins D1 and D2.

The IGF-I/PI3K signalling pathway is functional in myeloma cells with different IgH translocations

The relative insensitivity of t(11;14)-bearing MM cells to IGF-I and PI-103 might relate to an inability of IGF-I to activate the PI3K pathway in these cells. Thus, we examined the activation of key

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signalling molecules downstream of IGF-I and PI3K in representative HMCL. PKB is a major target of PI3K, mediating many of its functions, including cell cycle regulation and protection from apoptosis. Furthermore, PKB has been shown to positively regulate D-type cyclins, in part, by phosphorylation and inactivation of the Foxo family of transcriptional repressors.[18–21] Using an antibody specific for the Ser^{473}-phosphorylated (active) form of PKB, we observed robust activation of this pathway in response to IGF-I in MM1S, NCI-H929 and KMS12-BM cell lines, representing all three major IgH translocation groups (Figure 4). Phosphorylated PKB and/or phospho-Foxo proteins were detected after 10–30 min in all cases, and remained elevated for up to 24 h. Activation of PKB preceded the upregulation of cyclin D2 that was evident by 3 h in MM1S and by 6 h in NCI-H929, whereas cyclin D1 was unchanged in KMS12-BM. Importantly, PI-103 was able to completely block PKB activation in all the three HMCL tested, irrespective of the effect on D-type cyclin expression (Figure 4). In addition, phosphorylation (and inactivation) of Foxo3a and/or Foxo1/4 was also observed to be induced by IGF-I and inhibited by PI-103 in all the three HMCL. These data suggest that cyclin D1 expression in t(11;14) MM cells is independent of PI3K activation. In contrast, cyclin D2 induction in response to IGF-I in t(4;14) and t(14;16) cells depends on PI3K and correlates with PKB activation. In addition, these data suggest that the lack of responsiveness of t(11;14) cells to serum deprivation is unlikely to be due to the action of an autocrine growth factor loop, because most known MM mitogenic growth factors are dependent on PI3K signalling.

PI-103 inhibits IGF-I-mediated proliferation in primary myeloma cells bearing t(4;14) and t(14;16), and potentiates effects of anti-myeloma agents

The results of our experiments with HMCL indicated that the regulation of cyclin D1 in t(11;14)-bearing cells differs from that of cyclin D2 in cells harbouring either t(4;14) or t(14;16). The relative insensitivity of cyclin D1 levels to exogenous growth factors and to PI3K blockade in t(11;14)-bearing cells was also reflected in a lack of effect on other cell cycle proteins in these cells. We next sought to determine if these effects of PI-103 were representative of primary MM cells. $CD138^+$-selected MM cells from 12 patients with known IgH translocations (Figure 5) were cultured for 24 h in serum-free medium, either alone or in IGF-I ± PI-103. Figure 5a shows [^{3}H]-thymidine uptake in these cultures. Consistent with our data derived from HMCL, t(11;14) positive primary MM cells were unresponsive to IGF-I or PI-103 (left panel). Interestingly, case no. 2 showed a slight decrease in [^{3}H]-thymidine uptake in the presence of IGF-I, which was reversed by PI-103, an effect we have also observed in some t(11;14) HMCL. In contrast, 6/8 cases with either a t(4;14) or t(14;16) displayed increased [^{3}H]-thymidine incorporation with IGF-I, which was inhibited by PI-103 (Figure 5a, right panel). Notably, exposure to PI-103 led to a reduction in DNA synthesis in every case, including the two cases that had little/no response to IGF-I. The mean increase in [^{3}H]-thymidine uptake induced by IGF-I in these tumour cells with IgH translocations associated with cyclin D2 was 2.8-fold ($P<0.02$), which was reduced to 0.8-fold in the presence of PI-103 ($P<0.01$).

Analysis of cell cycle control proteins in these patient samples also confirmed our data from HMCL. In MM cells from patients harbouring t(14;16) or t(4;14), IGF-I increased expression of cyclin D2 and phospho-pRb, a response that was substantially down-regulated by PI-103 (Figure 5b, upper panel). In contrast, neither IGF-I nor PI-103 had any significant effect on cyclin D1 in MM cells from t(11;14) cases (Figure 5b, lower panel).

We next asked whether cell cycle inhibition might be potentiated when PI-103 is used in combination with common anti-myeloma agents. Primary $CD138^+$ myeloma cells were treated with PI-103 in the presence or absence of either melphalan, bortezomib, dexamethasone or lenalidomide. We observed that

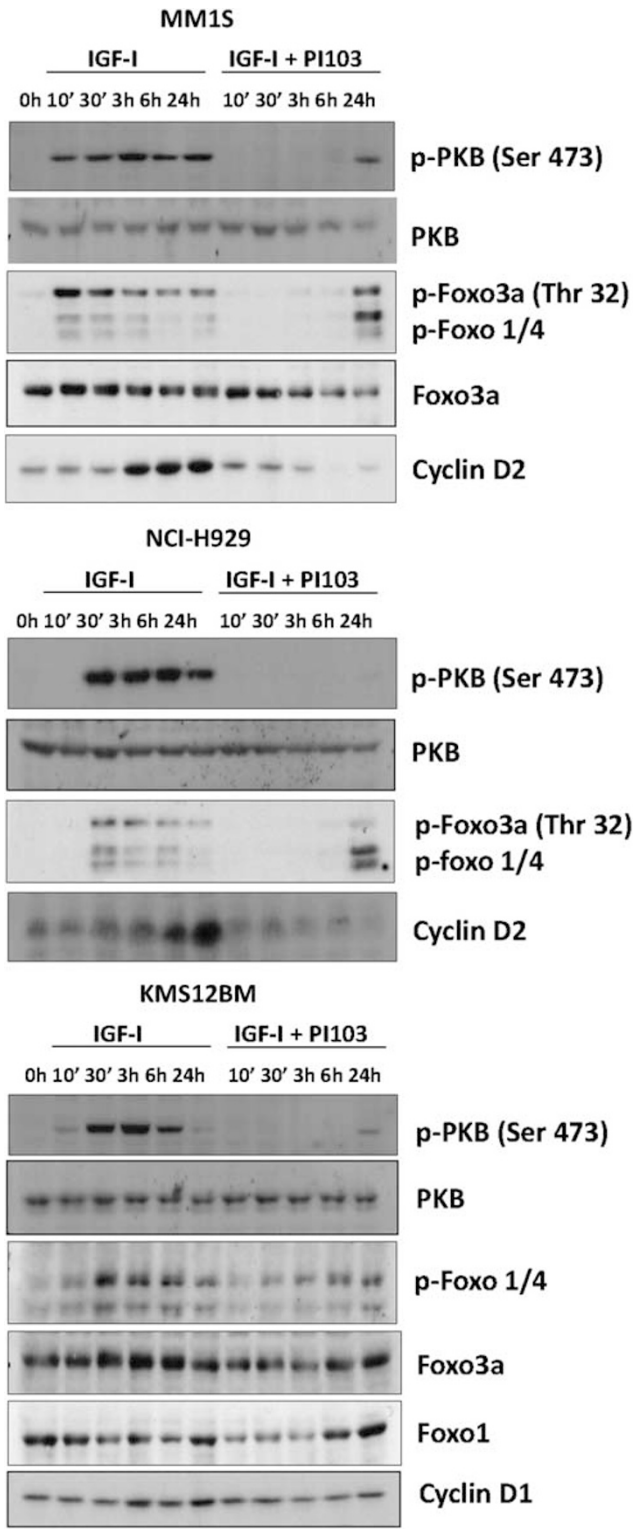

Figure 4. PI-103 blocks IGF-I-induced PI3K activation. MM1S, NCI-H929 and KMS12-BM cells were washed with serum-free medium (RPMI-1640) and stimulated with IGF-I (100 ng/ml) in serum-free medium for between 10 min and 24 h in the presence or absence of 1 μM PI-103. Western blots were probed with antibodies to PI3K phosphorylation target proteins and D-type cyclins, as indicated. Equivalent loading is demonstrated by the level of total PKB expression. NB: p-FOXO3a was not detected in KMS12-BM, whereas total FOXO3a was not detected in NCI-H929 and FOXO1 detected only in KMS12-BM.

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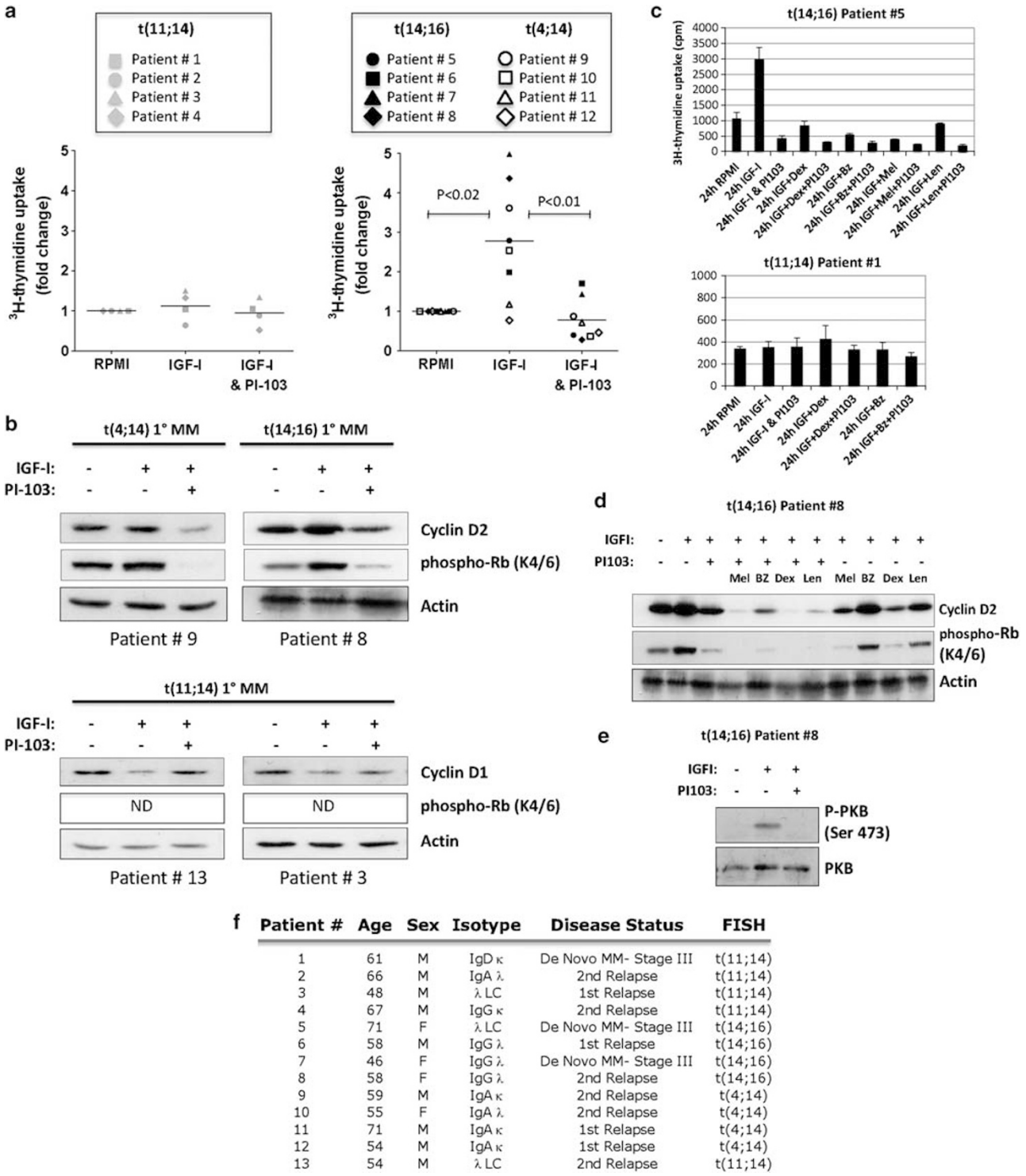

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Patient #	Age	Sex	Isotype	Disease Status	FISH
1	61	M	IgD κ	De Novo MM- Stage III	t(11;14)
2	66	M	IgA λ	2nd Relapse	t(11;14)
3	48	M	λ LC	1st Relapse	t(11;14)
4	67	M	IgG κ	2nd Relapse	t(11;14)
5	71	F	λ LC	De Novo MM- Stage III	t(14;16)
6	58	M	IgG λ	1st Relapse	t(14;16)
7	46	F	IgG λ	De Novo MM- Stage III	t(14;16)
8	58	F	IgG λ	2nd Relapse	t(14;16)
9	59	M	IgA κ	2nd Relapse	t(4;14)
10	55	F	IgA λ	2nd Relapse	t(4;14)
11	71	M	IgA κ	1st Relapse	t(4;14)
12	54	M	IgA κ	1st Relapse	t(4;14)
13	54	M	λ LC	2nd Relapse	t(11;14)

Figure 5. Effect of IGF-I and PI-103 on cell cycle progression in primary MM cells. (**a**) Proliferation assays were performed on selected CD138$^+$ MM cells cultured for 24 h in either serum-free medium (RPMI-1640) alone or with 100 ng/ml IGF-I ± 1 μM PI-103, as indicated. Each data point represents the mean fold change in [^{3}H]-thymidine uptake in triplicate wells for individual patients. The mean fold change in [^{3}H]-thymidine uptake for each group of patients for each culture condition is denoted by a horizontal bar. Data from cyclin D1-expressing t(11;14) patients ($n = 4$) are shown in the left-hand panel; those from cyclin D2-expressing t(4;14) and t(14;16) patients are in the right-hand panel. (**b**) Western blots of whole-cell lysates prepared from primary patient CD138$^+$ cells treated as described above, were probed with antibodies against cell cycle control proteins, as indicated. Anti-actin is shown as a loading control. (**c**) The effect of PI-103 in combination with chemotherapeutic drugs on [^{3}H]-thymidine uptake in two patients' CD138$^+$ cells, treated as described. (**d**) The effect of PI-103 in combination with chemotherapeutic drugs on the expression of cell cycle proteins in lysates prepared from cells from patient no. 8 treated as described above. (**e**) Primary CD138$^+$ cells were treated with 100 ng/ml IGF-I ± 1 μM PI-103 in serum-free RPMI-1640 medium for 15 min and analysed for PKB activation by western blotting of whole-cell lysates. Equivalent loading is demonstrated by total PKB expression. (**f**) Table showing details of patient samples used. BZ, bortezomib; Dex, dexamethasone; Len, lenalidomide; Mel, melphalan.

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the addition of PI-103 potentiates the cell cycle inhibitory effects of anti-myeloma agents in t(14;16)-bearing cells, but not in cells harbouring t(11;14) (Figure 5c). Addition of PI-103 led to a further reduction in [^{3}H]-thymidine uptake in MM cells from patient no. 5 (with t(14;16)), when compared with the effect of each anti-myeloma agent used alone. In contrast, there was no significant added effect of PI-103 with any of the anti-myeloma agents when added to t(11;14) positive cells from patient no. 1. Consistent with the potentiating effect on inhibition of DNA synthesis in t(14;16)-bearing cells, PI-103 also produced a greater reduction in phospho-pRb and cyclin D2 in MM cells from patient no. 8 when used in combination with any of the chemotherapeutic drugs compared with either agent used alone (Figure 5d). Furthermore, as seen in HMCL, PI-103 substantially inhibits IGF-I-mediated PKB phosphorylation in primary CD138$^+$ MM cells (Figure 5e), suggesting a mechanism by which cyclin D2 and subsequent cell cycle entry/progression are blocked by this agent.

Dual blockade of PI3K/mTOR with NVP-BEZ235 downregulates cyclin D2 and CDK6 *in vivo*

To test the effect of combined PI3K/mTOR blockade *in vivo*, we used the orally bioavailable inhibitor NVP-BEZ235.[16] We established subcutaneous tumours by injecting 10^7 cells from the HMCL JIM-1 that bears t(4;14) into the flank of unconditioned β2microglobulin null/NOD/SCID animals. Initial experiments had established the time course of tumour growth, and six animals were treated daily with intraperitoneal injections of NVP-BEZ235 once tumours were palpable. A control group of animals ($n = 5$) were injected with vehicle only. Animals were killed when the largest tumours had reached 1 cm at the longest diameter (4 weeks from tumour cell injection) and tumour weights were recorded. There was a trend to smaller tumours from animals treated with NVP-BEZ235 (Figure 6a), although this did not reach significance in this small series of animals. Importantly, there was a significant downregulation of pAkt, cyclin D2 and CDK6 in these tumours, when compared with tumours from control animals treated with vehicle (Figures 6b and c).

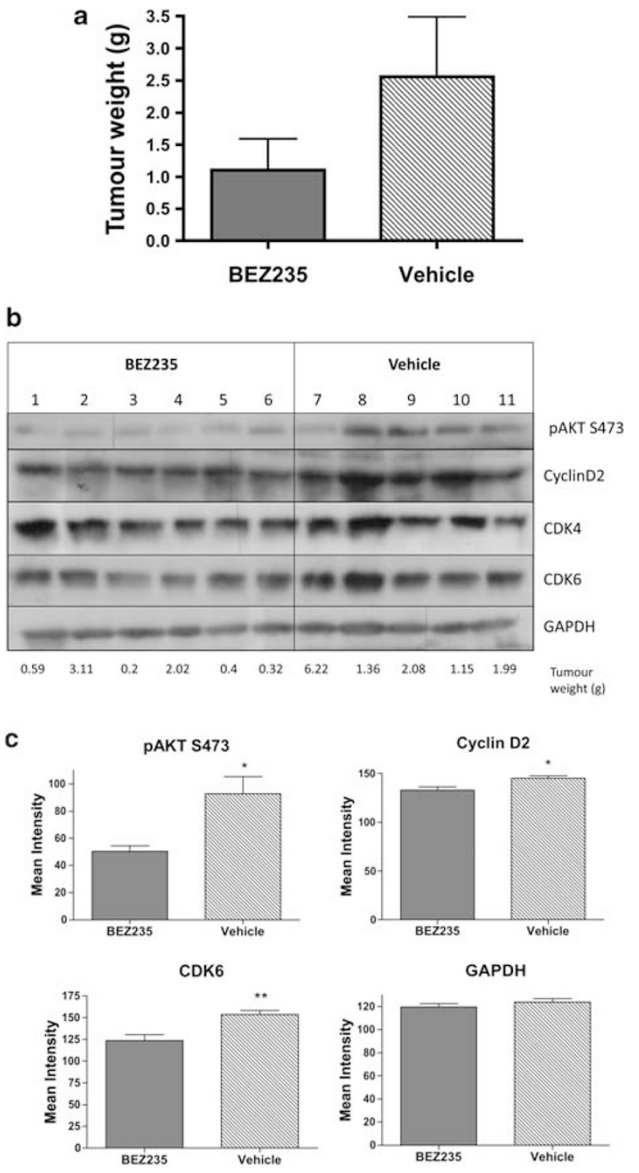

Figure 6. Effect of dual blockade of PI3K/mTOR*in vivo*. MM tumours were established in unconditioned β2microglobulin null/NOD/SCID animals by subcutaneous injection of JIM-1 cells (10^7/animal). Animals were randomly assigned to treatment (six) and control (five) groups; treatment groups received BEZ235 as detailed in Materials and methods, whereas control animals received injections of diluent only. Tumours were excised at 4 weeks, weighed and lysates probed for proteins as detailed below. (**a**) BEZ235 treated animals had smaller tumours than those from the control group. (**b**) Whole-cell lysates were prepared from about 30 mg of excised tumours. Blots were probed with antibodies against proteins as indicated. Anti-GAPDH is shown as a loading control. (**c**) Protein expression was quantified using Image J 1.43 and results are expressed as a ratio to control. *$P < 0.05$, **$P < 0.01$.

DISCUSSION

The major new finding reported here is that the effect of PI3K/mTOR blockade on cell cycle progression in MM cells is critically dependent on the underlying mode of cyclin D dysregulation, which in turn is dictated by IgH translocation status. The PI3K pathway is a major downstream signalling pathway utilised by the most important growth/survival factors for MM. Such factors, thought to have a role in mediating the protective effect of the myeloma BM microenvironment include IGF-I, IL-6, vascular endothelial growth factor, fibroblast growth factor, hepatocyte growth factor, APRIL and BAFF.[22,23] We and others have shown previously that in addition to its well-characterised role in mediating cell survival, PI3K is essential for mediating cell cycle entry and expression of D-type cyclins in B-lineage cells. Notably, mice that are genetically deficient in Class I PI3K are deficient in B-cell development and activation, and are unable to induce cyclin D2 normally in response to mitogens.[11,24] Furthermore, PI3K-knockout mice are phenotypically similar to mice lacking cyclin D2.[11,25]

Using a panel of HMCL with different IgH translocations, we observed that cells bearing t(4;14) or t(14;16) were more sensitive to PI-103-mediated growth inhibition when compared with t(11;14)-bearing cells. This differential sensitivity to PI-103 was apparent when cells were grown in their standard 10% FCS medium or in IGF-I alone. Further analysis revealed that these differences are largely due to differential effects on the cell cycle, with PI-103 treatment leading to cell cycle arrest in the t(4;14)- or t(14;16)-bearing cells, but not in t(11;14)-bearing cells. The cell cycle arrest observed in t(4;14) and t(14;16) cells was mediated by a decrease in cyclin D2, CDK 4 and CDK6 expression and an increase in levels of p27, concomitant with decreased CDK4/6-specific pRb phosphorylation and total pRb. These changes are similar to those observed in normal B lymphocytes undergoing cell cycle arrest due to serum deprivation or PI3K inhibition,[24,26,27] where decreased cyclin D2-CDK4/6 complexes are unable to titrate p27 (a CIP/WAF family CKI) away from cyclin E/CDK2 complexes, leading to further dephosphorylation of pRb and

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resultant repression of the E2F transcription factor. Total pRb is also downregulated as it is itself an E2F-regulated gene. In contrast to cyclin D2-expressing cells, and in keeping with the lack of cell cycle inhibition, there were little or no changes observed in G1-phase cell cycle control proteins in t(11;14) cells treated with PI-103.

Here, we show, for the first time, that PI3K inhibition modulates cyclin D2 protein levels in primary CD138+ MM cells. We also confirm, in a larger cohort of patients, our earlier findings that, in primary MM cells bearing t(4;14) or t(14;16), cyclin D2 is functionally upregulated by IGF-1, unlike cyclin D1 expressed via a t(11;14), which is unresponsive to IGF-1. IGF-I stimulation increased protein levels of cyclin D2 as well as CDK4/6-specific pRb phosphorylation in MM cells from patients harbouring t(4;14) or t(14;16). We have recently reported that APRIL modulates cyclin D2 and cell cycle proteins to induce cell cycle progression in t(4;14)- and t(14;16)-bearing primary MM cells.[8] Such cell cycle responses to APRIL were absent in cyclin D1MM cells. In t(4;14) or t(14;16) cells, cyclin D2 is upregulated indirectly by overexpression of the MMSET/FGFR3 proteins or by the c-MAF transcription factors, which may themselves be subjected to regulation by mitogens. Thus, it is unsurprising that cyclin D2 which is under the control of its natural, growth-factor responsive promoter is sensitive to PI3K inhibition, because PI3K is known to be a major regulator of this protein in haematopoietic cells. MM cells carrying a t(11;14), however, express cyclin D1 constitutively by the strong IgH enhancer elements, thus rendering it independent of mitogenic signalling. What is perhaps surprising is that other cell cycle proteins, such as CDK4/6, total pRb and p27 are also unaffected by mitogens and/or PI3K inhibition in t(11;14) cells, because expression of these proteins should remain under normal physiological regulation. This is especially pertinent for p27, which is known to be regulated by PI3K.[28,29] It is possible that in the presence of excess cyclin D1, the cyclin D1-CDK4/6-p27 complexes are stably maintained and/or exert feedback signalling that overrides regulation by mitogenic withdrawal or PI3K inhibition. Alternatively, t(11;14) MM cells may evolve in a mitogen-independent manner, so that upregulation of cyclin D1 (an early, premalignant event) is associated with later mutations that maintain constitutive expression of CDK4/6-p27 complexes independently of PI3K activity. However, because most primary t(11;14) MM cells are not constitutively in cycle,[30] there must be as yet unrecognised signals that regulate their cell cycle entry *in vivo*.

Although this study has focussed on MM cells harbouring an IgH translocation, approximately 50% of MM cases are characterised by hyperdiploidy and an absence of an IgH translocation. It is technically challenging to investigate the mechanisms of cell cycle control in this group, because they are not adequately represented by HMCL. However, recent data from our laboratory, utilising an improved culture method for primary MM cells, have demonstrated that in the hyperdiploid group, cyclin D2-expressing tumours are more sensitive to growth factors than cyclin D1-expressing tumours, but are less responsive when compared with cyclin D2-expressing cases harbouring t(14;16) or t(4;14).[8] This study has focussed on cell cycle regulation, however, other work from our group has shown that dependence on survival signalling from the PI3K pathway is also dictated by IgH translocation status.[31] These data support the hypothesis that t(11;14) cells clonally evolve in a manner that makes them relatively refractory to signals emanating from these classical myeloma growth/survival factors when compared with those harbouring IgH translocations targeting cyclin D2.

The relative insensitivity of t(11;14) cells to PI-103 could be explained by a functionally inactive PI3K pathway. Alternatively, they might have a hyper-activated pathway (for example, due to mutation of PI3K subunits) refractory to enzymatic inhibition. Neither of these explanations, however, is borne out by our observations on the activation of key PI3K signalling intermediates. When we compared MM cells representing the three major IgH translocation groups, we found no differences in IGF-I-induced activation of PKB. Furthermore, PI-103 abrogation of PKB phosphorylation was equally apparent in t(11;14) cells compared with t(4;14) or t(14;16) cells. Thus, the differential impact of PI3K/mTOR blockade on cell cycle progression between cyclin D2-expressing t(4;14) or t(14;16) and cyclin D1-expressing t(11;14) MM tumours may be due to the composition and activity of cyclin D–CDK complexes, and their regulation by proteolytic mechanisms. Differential sensitivity to PKB inhibition has been observed by Zollinger *et al.*,[32] who attributed this to differences in constitutive PKB phosphorylation both in HMCL and primary MM cells. Although the emphasis was on apoptosis rather than cell cycle regulation, the HMCL shown to be sensitive to PKB inhibition were OPM2 and MM1S, which are t(4;14) and t(14;16) positive, respectively, whereas the t(11;14) HMCL, U266 was insensitive. These, and our findings reported here, raise the possibility that IgH/cyclin D status may dictate patients' response to therapies aimed at inhibition of PKB and other targets downstream of PI3K. Several genetic abnormalities have been associated with increased PKB activation, including mono-allelic deletion of PTEN, present in approximately 5% of BM MM cells, and up to 20% in plasma cell leukaemias and HMCL.[33] However, PTEN mutations were found to be absent in most MM cells with constitutively activated PKB, while 'hotspot' mutations in PIK3CA (which codes for the p110 alpha catalytic subunit of PI3K) and PKB identified in a variety of other tumours were not found in MM,[34] suggesting either that other genetic abnormalities are responsible or that the activation is due to increased growth factor responsiveness.

We used primary MM cells to test whether the inhibitory effect of PI-103 on cell cycle progression would enhance the activity of anti-MM agents. We observed, in primary t(14;16)-bearing MM cells, that the addition of PI-103 to melphalan, bortezomib, dexamethasone or lenalidomide resulted in a greater inhibition of DNA synthesis and greater reduction in levels of cyclin D2 and CDK4-/6-phosphorylated pRb than observed with either drug alone. In contrast, in t(11;14)-bearing cells, no such potentiating effect was observed. These data indicate that combination therapies utilising PI3K/mTOR inhibitors would be particularly effective in those patients with IgH translocations that target cyclin D2. The importance of dual inhibition of both PI3K and mTOR pathways has been recently recognized.[14] Although PI-103 itself is unlikely to progress to clinical development because of its limited solubility and extensive metabolism, several pharmacologically optimised PI3K/mTOR inhibitors are in development. One such agent, NVP-BEZ235, was also recently shown to inhibit MM cell growth and proliferation and exhibit synergy with anti-MM drugs in two independent studies.[16,35] McMillin *et al.*,[35] in their work on HMCL, noted that there was varying sensitivity to the effect of this PI3K/mTOR inhibitor, but did not report any correlation with genetic lesion. Using NVP-BEZ235, we demonstrate that dual blockade of PI3K/mTOR downregulates cyclin D2 and CDK6 *in vivo*, leading to an attenuation of tumour growth in MM cells bearing t(4;14).

In summary, we report that the effect of PI3K/mTOR inhibition on cell cycle progression in MM cells segregates with IgH/D-type cyclin status. Importantly, tumour cells from patient subgroups with inferior outcomes, and characterised by higher proliferative behaviour, including responses to mitogens, are particularly sensitive to PI3K pathway blockade. These tumour cells express cyclin D2 in conjunction with the t(4;14) or t(14;16), whereas tumours with a different genetic background, that is, expressing cyclin D1 with t(11;14), that confers a neutral prognosis, are largely insensitive. Our findings provide the rationale for the incorporation of dual PI3K/mTOR inhibitors in treatment strategies aimed at patients with t(4;14) or t(14;16), and set the scene for future mechanistic studies on cell cycle regulation in these distinct subtypes of MM.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by Kay Kendall Leukemia Fund (JG), Cancer Research United Kingdom (DK, CS and JQ) and the UCLH/UCL Comprehensive Biomedical Research Centre (KLY, AK).

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