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The polo-like kinase inhibitor BI 2536 exhibits potent activity against malignant plasma cells and represents a novel therapy in multiple myeloma

Helen J. Stewart, Lyudmila Kishikova, Fiona L. Powell, Sally P. Wheatley, and Timothy J. Chevassut

Brighton and Sussex Medical School, University of Sussex, Falmer, Brighton, East Sussex, UK

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Objective. Polo-like kinase 1 (Plk1) is a regulator of the cell cycle that has been implicated in the pathology of many cancers. We have investigated whether this kinase plays a role in multiple myeloma (MM) using the Plk1 inhibitor BI 2536.

Materials and Methods. We have used six MM cell lines and six patient-derived samples to determine the effects of the Plk1 inhibitor, BI 2536, on cell viability, apoptosis, and cytokinesis. We have also examined the effect of the microenvironment on these parameters and the effects of BI 2536 in combination with other antimyeloma agents.

Results. We show that MM cell lines and patient samples express *PLK1* and that cell death by apoptosis occurs when Plk1 is inhibited. Cells treated with BI 2536 accumulate in the G₂/M phase of the cell cycle causing endoduplication. The effects of BI 2536 are not abrogated when cells are cultured on extracellular matrix components, in the presence of interleukin-6, or with bone marrow stromal cells.

Conclusions. Plk1 inhibition leads to cell death in MM cell lines and patient myeloma samples. Our data suggest that inhibition of Plk1 may have potential use as a therapeutic strategy in multiple myeloma. © 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Polo-like kinase 1 (Plk1) is one of four polo-like kinase proteins with different subcellular locations and functions involved in cell-cycle regulation, particularly during G₂ and mitosis, and it also plays an important role in inhibiting apoptosis [1]. The polo-like kinases belong to a family of mitotic serine/threonine kinases, which contain one or two C-terminal regions, known as polo box domains, and a highly conserved N-terminal kinase domain. Plk1 is highly expressed in malignant cells and serves as a negative prognostic marker in many human cancer types, including hematological cancers [2,3]. Forced expression of Plk1 results in multinucleation, which is a feature of many cancer cells and characteristic of chromosomal instability.

The role of polo-like kinases in multiple myeloma (MM) has yet to be addressed. MM is characterized by the malignant growth of immunoglobulin-producing plasma cells, mainly in the bone marrow. Despite the introduction of

proteasome inhibitors and immunomodulatory agents in the treatment of this condition, patients invariably relapse, highlighting the need for developing alternative treatment strategies. The observation that the proliferation rate of MM cells tends to rise with progression of the disease suggests that targeting the polo-like kinases may represent an attractive therapeutic target.

This study shows that the Plk1 inhibitor BI 2536 [4] effectively kills MM cells in a time- and dose-dependent manner and that this effect is not influenced by components of the extracellular matrix (ECM), interleukin-6 (IL-6), or bone marrow stromal cells. Moreover, the action of BI 2536 is potentiated when combined with bortezomib and dexamethasone, revealing synergy with these drugs. Our results suggest that inhibition of Plk1 represents an attractive therapeutic strategy in the treatment of MM.

Materials and methods

Materials

BI 2536 was obtained from Axon Medchem (Groningen, The Netherlands). Bortezomib was obtained from LC Laboratories (Boston, MA, USA). Dexamethasone, Histopaque 1077, ECM,

Drs. Stewart and Kishikova contributed equally to this work as first co-authors.

Offprint requests to: Timothy J. Chevassut, M.D., Ph.D., Brighton and Sussex Medical School, University of Sussex, Falmer, Brighton, East Sussex BN1 9PS, UK; E-mail: t.chevassut@bsms.ac.uk

laminin, and fibronectin were obtained from Sigma (Poole, UK). Penicillin, streptomycin, L-glutamine, RPMI 1640, Trizol, and reverse transcription polymerase chain reaction (PCR) primers were purchased from Invitrogen (Paisley, UK). Fetal calf serum was purchased from Biosera (Ringmer, UK). IL-6 was purchased from Peprotech (London, UK). Celltiter-Glo kit and Caspase-Glo 8 kit were purchased from Promega (Southampton, UK). Taq polymerase and human reference RNA were purchased from Stratagene. CD138 microbeads were purchased from Miltenyi Biotec (Bisley, UK). WST-1 reagent was purchased from Roche (Burgess Hill, UK). Annexin-V/propidium iodide kit was purchased from BD Biosciences (Oxford, UK). Quantitech cDNA kit was purchased from Qiagen (Crawley, UK).

Cell lines and primary samples

A total of six human MM cell lines were used in this work: MM.1S, RPMI-8226, U-266, MM1R, KMS-12, and MOLP-8. These cells were a kind gift from Dr. E. Ocio (University of Salamanca), Drs. S. Sahota and B. Guinn (University of Southampton), and Dr. Y. Calle (King's College Hospital, London). The integrity of these cell lines was assessed by protein electrophoresis of cell supernatants to confirm production of the expected type of monoclonal immunoglobulin. Enhanced green fluorescent protein MM.1S cells and cherry red—expressing HS-5 stromal cells were from Dr. Calle [5]. Untransfected HS-5 stromal cells were from Dr. Ocio. Cells were cultured in RPMI-1640 (or Dulbecco's modified Eagle's medium, in the case of HS-5 cells) supplemented with 10% fetal calf serum, 100 mM L-glutamine, penicillin (100 IU/mL), and streptomycin (100 µg/mL).

A total of six myeloma patient samples were studied. All primary bone marrow aspirates were taken from routine diagnostic specimens after informed consent of the patients. The project received approval from the local ethics committee (The Brighton Blood Disorder Study, references 09/025/CHE and 09/H1107/1) and was conducted in accordance with the Declaration of Helsinki. Mononuclear cells were isolated by Histopaque 1077 density gradient purification [6]. CD138⁺ plasma cells were isolated from the mononuclear cell preparations by positive selection using CD138 magnetic microbeads, according to manufacturer's instructions. Purity of the cells was checked by flow cytometry.

Reverse transcription PCR

Total RNA was extracted from cell lines (5×10^6 cells) using Trizol reagent according to manufacturer's instructions. RNA (1 µg) was reverse transcribed using a Quantitech cDNA kit. Human reference RNA was used as a positive control. Primers for human *PLK* genes were as follows [7]: *PLK1* Forward 5'-TGACGGCACTGAGTCC TACC-3', Reverse 5'-AGGCCTTGAGACGGTTGCTG-3'; *PLK2* Forward 5'-TCGGCTCTACCTCCTCAGT-3', Reverse 5'-GTTCT GGTTCACACAGTCC-3'; *PLK3* Forward 5'-CCACTTTGAGG ACGCTGACA-3', Reverse 5'-CTGCTGATAGGGAGTCGATC-3'; *PLK4* Forward 5'-ACCACCCTCACCTACTGACA-3', Reverse 5'-GTGCCTGTGTTGGAGAAGCA-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Forward 5'-AGGGGTCTACATGGCAA CTG-3', Reverse: 5'-CGACCACTTTGTCAAGCTCA-3'. The PCR cycles were as follows: *PLK1* (35 cycles, 440 bp), *PLK2* (30 cycles, 420 bp), *PLK3* (35 cycles, 580 bp), *PLK4* (35 cycles 600 bp), GAPDH 30 cycles, 228 bp. Each cycle consisted of denaturation (94°C for 15 seconds), annealing (30 seconds), and extension (72°C for 30 seconds). The annealing temperatures were as follows: 60°C for

PLK1, 57°C for *PLK2*, 56°C for *PLK3*, 55°C for *PLK4*, 54°C for GAPDH. The first cycle was preceded by a denaturation step of 3 minutes at 94°C and the last one was followed by an extension step of 3 minutes at 72°C. The resulting PCR fragments were *PLK1* (440 bp), *PLK2* (420 bp), *PLK3* (600 bp), *PLK4* (580 bp), GAPDH (228 bp), and were run on 1.5% agarose gels containing Gel Red and visualized under ultraviolet light.

Cell viability assays

For cell lines, cell viability was assessed using a WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay. The 5×10^4 cells plated per well in 96-well plates were incubated in the presence of drug for the times indicated. For patient samples, 1×10^4 cells were plated in 96-well plates and cell viability measured using a luminescent Celltiter-Glo kit according to manufacturer's instructions. The viability of untreated cells was set as 100% and viability in other groups was calculated by comparing the optical density readings with the control. BI 2536 was dissolved in dimethyl sulfoxide and stored at -20°C. Dilutions in phosphate-buffered saline (PBS) were used for all experiments.

Growth curves

MM cells were plated at a density of 5×10^4 cells per well in a 24-well plate in 400 µL cell culture medium in the presence or absence of 100 nM BI 2536. PBS was added to control wells. Every 24 hours for 72 hours, cells were counted in the presence of trypan blue (to determine cell viability) and the results recorded.

Coculture experiments

HS-5 bone marrow stromal cells (cherry red—expressing) were plated onto 13-mm glass coverslips and grown in RPMI + 10% fetal calf serum until confluent. Fifty thousand enhanced green fluorescent protein—MM.1S cells were seeded onto the HS-5 cells [5] and cultured in the presence or absence of BI 2536 for 24 hours. After this time, coverslips were fixed in 4% paraformaldehyde for 10 minutes and stained with 4,6-diamidino-2-phenylindole. After a final wash through PBS, coverslips were mounted in Citifluor anti-fade mounting medium and viewed for immunofluorescence using a Leica DM 5000B microscope fitted with a Leica DPC300FX digital camera. The number of green fluorescing MM.1S cells was counted in images of five random fields taken at 5× magnification.

Cell adhesion to laminin, fibronectin, and ECM

Laminin and fibronectin (10 µg/mL) were applied to a poly-L-lysine—coated 96-well plate for 1 hour at room temperature. The substrates were aspirated and immediately replaced with medium containing the indicated number of cells. ECM solution was obtained by dissolving ECM in ice-cold PBS. The ice-cold ECM solution (50 µL) was then pipetted into the wells of 96-well tissue culture plates. Gelling of the ECM coating of the well was achieved by incubation at 37°C, as described previously [6]. Cells were plated directly onto the gelled ECM.

Apoptosis assays

BI 2536—induced apoptosis of MM cell lines and a patient sample were assayed using an Annexin-V/propidium iodide kit according to manufacturer's instructions. Analysis of Annexin-V/propidium iodide staining by flow cytometry was carried out using a Becton Dickinson LSR II with data acquisition software FACS Diva.

Activation of caspase-8 was measured using a Caspase-Glo 8 assay (Promega). Cells were plated in black 96-well plates at a density of 10,000 cells per well and treated with 100 nM BI 2536 for 24 hours.

Immunolabeling

MM cell lines were plated onto poly-L-lysine-coated coverslips for 24 hours, then treated with 100 nM BI 2536 for an additional 48 hours. After this time, coverslips were fixed in 4% paraformaldehyde for 10 minutes, rinsed through PBS, then incubated in fluorescein isothiocyanate-conjugated rabbit anti-Annexin-V together with propidium iodide for 30 minutes at room temperature. After a final wash through PBS, coverslips were mounted in Citifluor anti-fade mounting medium and viewed for immunofluorescence using a Leica DM 5000B microscope fitted with a Leica DPC300FX digital camera.

Statistical analysis

All values are shown as mean \pm standard error of mean from at least three independent experiments (or a representative experiment of three is shown) and considered significant if $p < 0.05$. Significance between groups was calculated using Student's t-tests.

Results

PLK genes are present in MM cells

We initially carried out experiments to determine which of the *PLK* messenger RNAs were detectable in MM cells. U-266, MM.1S, and RPMI-8226 were used for this purpose. The results confirm that these cell lines express all four *PLK* genes (Fig. 1A). Because inhibitors of Plk1 are readily available and Plk1 expression is associated with malignant progression, we chose to continue our study focusing on this enzyme.

Expression of *PLK1* was assessed in five primary myeloma cell samples by reverse transcription PCR using RNA extracted from bone marrow plasma cells. *PLK1* expression was detected in all samples, suggesting that Plk1 is a legitimate therapeutic target in myeloma (Fig. 1B). By contrast, we did not detect *PLK1* expression in normal bone marrow cells consistent with available microarray results (UCSC genome bioinformatics Website) obtained using GNF gene expression atlas 2 data from U133A and GNF1H chips.

BI 2536 induces cytotoxicity in MM cell lines and patient samples in a dose- and time-dependent manner

In order to determine the effect of inhibition of Plk1 on myeloma cells, we treated MM cell lines (Fig. 2A) and CD138⁺ plasma cells from patient samples (Fig. 2B) with varying doses of BI 2536 (0.001–100 μ M), a potent and highly specific inhibitor of Plk1, for a 48-hour period. Cell viability was assessed using WST-1 or a luciferase-based assay (Celltiter-Glo). IC₅₀ values for the MM cell lines RPMI-8226 and MM.1S were ≤ 10 nM, which were significantly lower than the IC₅₀ values from nonmalignant

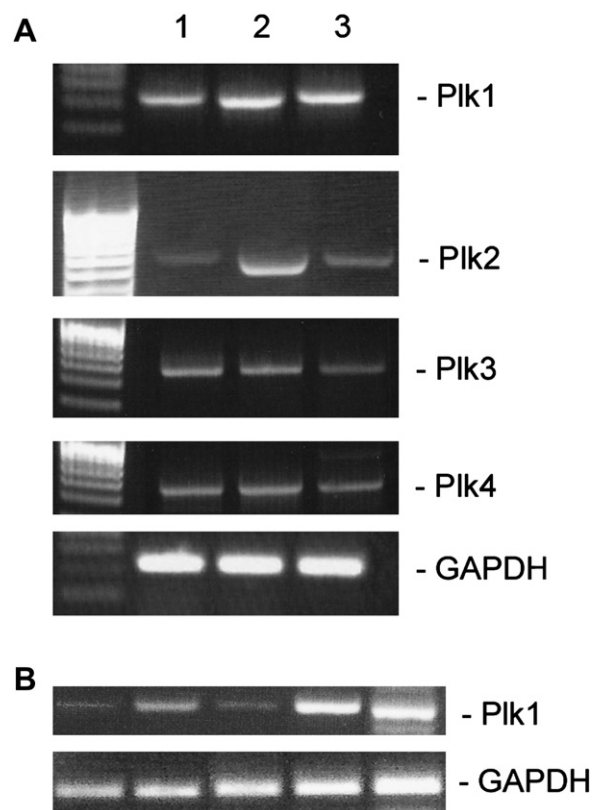


Figure 1. Multiple myeloma cell lines express and patient samples express *PLK* genes. Reverse transcription PCR was carried out on complementary DNA prepared from 1 μ g RNA from three cell lines and five patient samples. (A) Lane 1: MM.1S, Lane 2: RPMI-8226, Lane 3: U-266. (B) Patient samples.

tissues. The U-266 cell line had a higher IC₅₀ value of 1 to 10 μ M, which was similar to the IC₅₀ value of CD138⁺ plasma cells from newly diagnosed MM patients, also 1 to 10 μ M. Peripheral blood mononuclear cells (data not shown) and confluent cultures of HS-5 cells (Fig. 2C) had IC₅₀ values of ~ 100 μ M.

Examination of the effect of 100 nM BI 2536 on MM cell lines during a 72-hour period showed that nearly all cells had died/arrested during this time (Fig. 2D). Cell death/arrest is induced in around 50% of cells from all cell lines by 48 hours. To establish that inhibition of cell growth was occurring, we carried out growth curves in the presence of 100 nM BI 2536 using MM cell lines. Growth curves of MM cell lines show that in the presence of BI 2536 cell growth is inhibited (Fig. 2E and F).

BI 2536 induces MM cell cytotoxicity in the presence of ECM components, IL-6, and bone marrow stromal cells

As the activity of MM cells is known to be regulated by the bone marrow microenvironment [8,9], we sought to determine the effect of this environment on the response of MM cells to BI 2536. We used ECM, laminin, and fibronectin as cell culture substrates. Treatment of MM cells

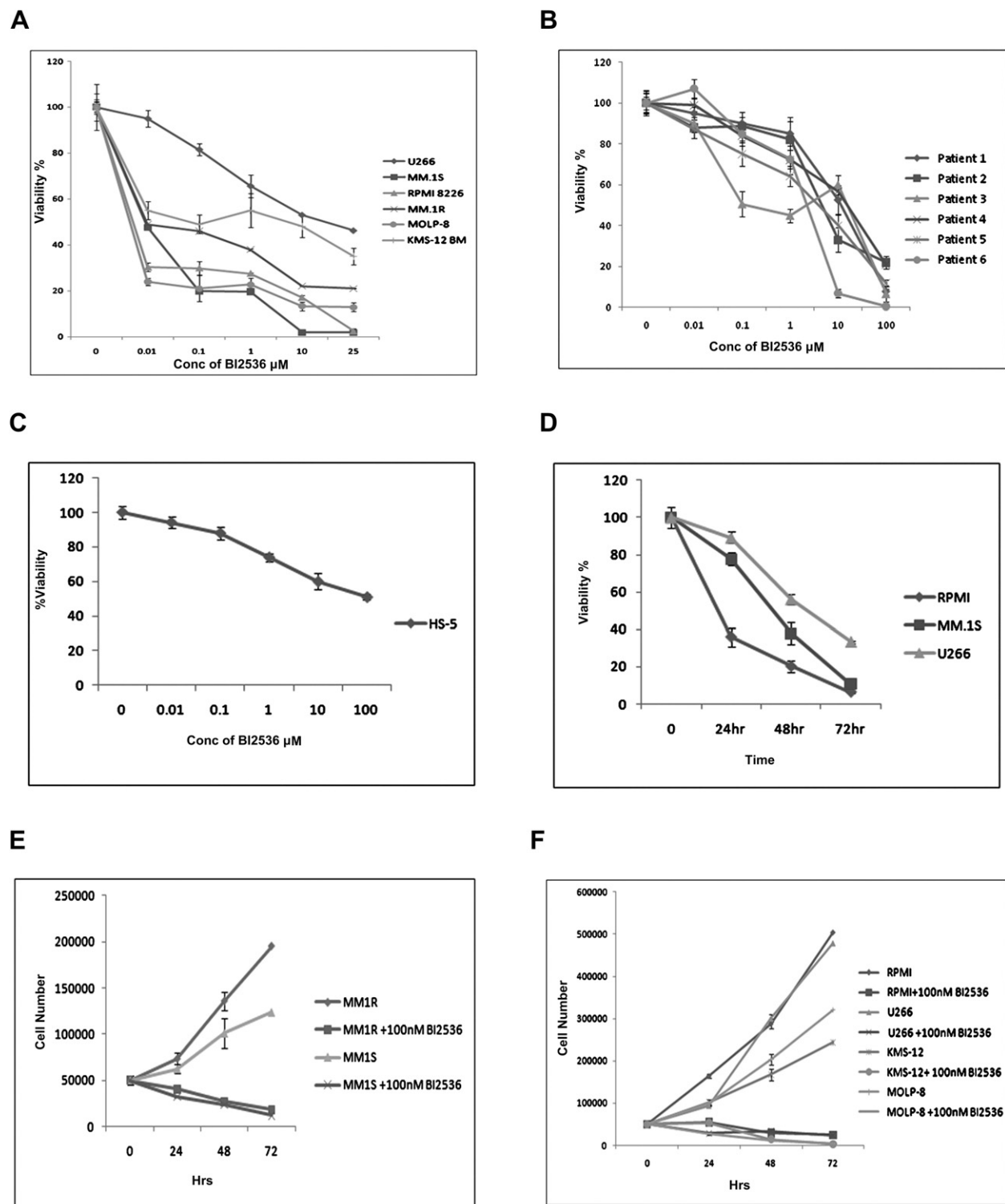


Figure 2. The Plk inhibitor BI 2536 inhibits MM cell viability in a dose-dependent manner. Cell lines (A) and patient samples (B) were treated with various doses of BI 2536 for 48 hours and cell viability assessed. Confluent HS-5 cells are less responsive to BI 2536 (C). A time-response curve to 100 nM BI 2536 is shown (D). Growth curves for MM cells during a 5-day period (E, F). Data are representative of at least three independent experiments performed in triplicate.

on these substrates with 100 nM BI 2536 did not protect the cells from cell death (Fig. 3A). Furthermore, the *in vitro* activity of BI 2536 was not suppressed by IL-6, a potent MM cell survival factor, or in the presence of ECM together

with IL-6 (Fig. 3B). In additional experiments, we cultured MM.1S cells expressing green fluorescent protein on monolayers of HS-5 bone marrow stromal cells. Treatment of these cells with 100 nM BI 2536 did not protect the cells

from cell death, showing that BI 2536 is active even in the presence of stromal cells.

BI 2536 induces apoptosis and endoduplication in MM cells

In order to determine whether the decrease in cell viability seen in MM cells treated with BI 2536 was due to apoptosis, we assessed the presence of apoptotic cells by measuring Annexin-V staining and propidium iodide uptake in MM cells by flow cytometry. Results showed BI 2536 caused a dose-dependent induction of apoptosis

in both cell lines (Fig. 4A) and a patient sample (not shown) within 24 hours. These results were confirmed by immunolabeling cells cultured in the presence of 100 nM BI 2536 with anti-Annexin-V (Fig. 4B). To confirm that BI 2536 was preventing cytokinesis in MM cell lines, we permeabilized the cells and labeled with propidium iodide. Flow cytometry revealed that BI 2536 results in an increased proportion of 4N cells and also a population of 8N cells indicative of endoreduplication (Fig. 4C). This occurs when G₂/M cells fail to separate after DNA synthesis and proceed to a further cycle. Analysis of the mechanism of

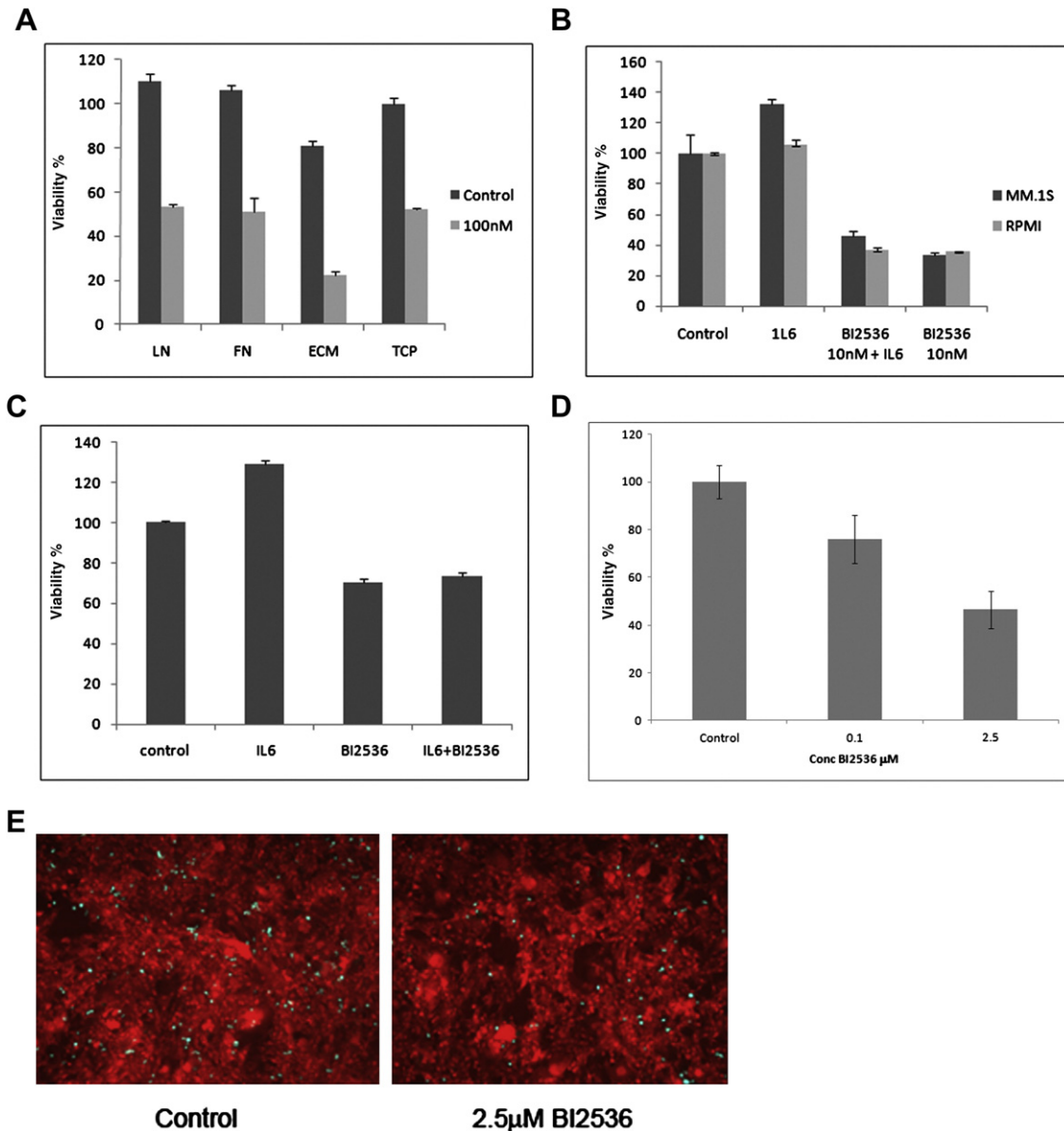


Figure 3. BI 2536 can overcome the protective effects of the bone marrow microenvironment. (A) MM cell lines (50,000 cells per well) were cultured on laminin (LN), fibronectin (FN), extracellular matrix (ECM), or tissue culture plastic (TCP) in the presence or absence of 100 nM BI 2536. (B) MM cells cultured on TCP in the presence of 10 ng/mL IL-6, 100 nM BI 2536, or IL-6 + 100 nM BI 2536. (C) MM cells cultured on ECM in the presence of 10 ng/mL IL-6, 100 nM BI 2536, or IL-6 + BI 2536. (D) Enhanced green fluorescent protein expressing MM.1S cells cultured on mCherry-expressing bone marrow stromal cells in the presence or absence of BI 2536 for 24 hours. (E) Photomicrographs of cell cultures. Data shown are from a representative experiment on the RPMI-8226 cell line.

apoptosis in these cells revealed that caspase-8 activation had occurred (Fig. 4D).

BI 2536 shows synergy with bortezomib and dexamethasone

Bortezomib and dexamethasone are routinely used in treatment of MM. Therefore, it was of interest to determine whether inhibition of Plk1 would potentiate antimyeloma activity of these drugs. We combined doses of drugs that caused <50% inhibition of cell viability when used alone.

Our results showed that when MM cell lines (RPMI-8226, U266 and MM.1S) were treated with BI 2536 combined with either bortezomib or dexamethasone, there was potentiation of cell killing indicative of drug synergy (Fig. 5).

Discussion

This study shows that all four polo-like kinase genes are expressed in MM cell lines and that inhibition of one of these, Plk1, results in MM cell death by apoptosis. Plk1 was also

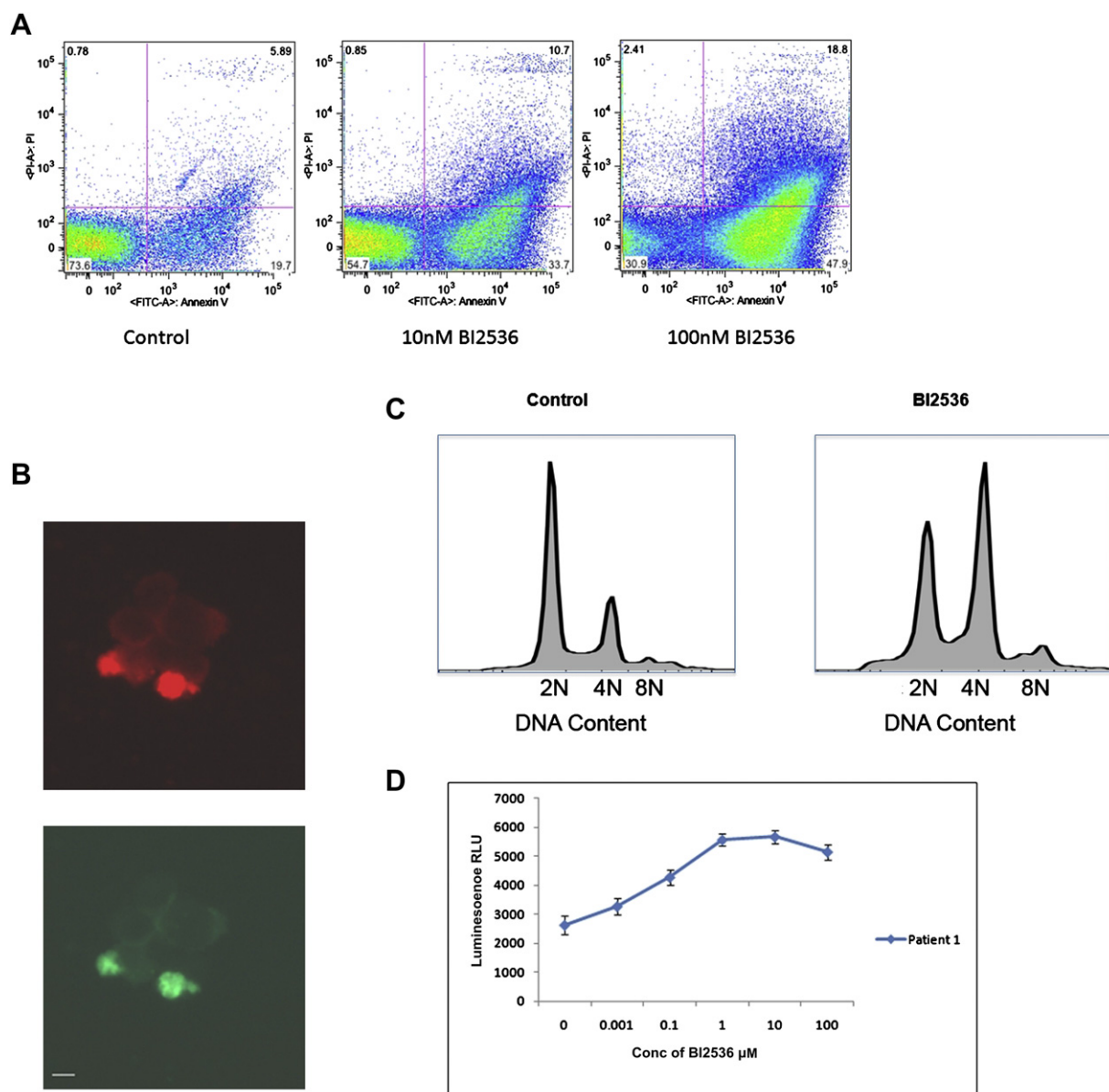


Figure 4. BI 2536 causes apoptosis, endoduplication, and caspase-8 activation in MM cells. **(A)** MM.1S cells were treated with 10 nM and 100 nM BI 2536 for 24 hours then analyzed for Annexin-V and propidium iodide expression by flow cytometry. **(B)** Cells were plated in 100 nM BI 2536 for 48 hours then immunolabeled using fluorescein isothiocyanate-linked anti-Annexin-V and stained with propidium iodide. Photos were taken using a Leica DM 5000B microscope fitted with a Leica DPC300FX digital camera using a $\times 40$ objective. Images were captured using Leica application suite software. Scale bar: 10 μ m. **(C)** MM.1S cells were analyzed by propidium iodide (PI) cell-cycle analysis. This reveals an increased proportion of 4N cells in G₂/M and a population of 8N cells indicative of endoreduplication by 24 hours. **(D)** CD138⁺ patient cells were plated at a density of 10,000 cells per well in 96-well plates and assayed for caspase-8 activation.

found to be present in patient samples. Plk1 is the only isoform shown to be involved in the activation of Cdk1, chromosome segregation, centrosome maturation, bipolar spindle formation, and execution of cytokinesis [1]. Plk1 activity is elevated in tissues and cells with a high mitotic index and Plk1 is often overexpressed in tumors, suggesting that it may be a highly suitable target for cancer therapy [10]. About 80% of human tumors of various origins express high levels of Plk1 transcripts. However, Plk1 messenger RNA is mostly absent in surrounding healthy tissues [11]. Plk2 and Plk3 regulate G₁ and early S phase entry [12,13], while Plk3 is downregulated in some cancers [14]. Plk4 functions in centriole duplication [15].

In this study, we show that the Plk1 inhibitor BI 2536 induces cell death by apoptosis in both cell lines and patient samples. BI 2536 treatment results in accumulation of 4N cells arrested at G₂/M and a population of 8N cells indicative of endoduplication. Interestingly, the sensitivity of primary patient samples prepared from bone marrow aspirates is lower than that observed for cell lines (1–10 μ M for primary MM cells vs. 1–10 nM for most cell lines). We speculate that this is attributable to a lower mitotic rate in primary MM cells and also the U266 cell line that had a higher than expected IC₅₀ level. We predict that cells from relapsed MM patients, which have shorter tumor-doubling times, would exhibit greater drug sensitivity [16].

The IC₅₀ level for the primary MM cells was in the range of 1 to 10 μ M. In phase I, dose-escalation and pharmacokinetic studies of BI 2536 in patients with advanced solid tumors, the maximum tolerated dose was 200 mg, which would produce an estimated tissue concentration of approximately 10 μ M (consistent with the measured plasma concentrations of 1–10 μ M) [17]. BI 2536 is currently in

phase II/III clinical studies, although this is not the only Plk1 inhibitor under development. Several others, such as BI 6727, are in phase I trials with other inhibitors in preclinical development [18].

Interestingly, we observed that cell viability, as measured by proliferation growth curves, was blocked at lower concentrations of BI 2536, for instance, 100 nM vs. 10 μ M for the cell line U266. This indicates that cell-cycle arrest occurs at lower concentrations than required for inducing apoptosis. Thus, BI 2536 is cytostatic at low doses and cytotoxic at high doses, suggesting that it might be well suited to synergistic activity when combined with other antimyeloma agents.

In healthy tissues where cell division is minimal, such as peripheral blood mononuclear cells and confluent cultures of the stromal cell line HS-5, there is a limited reduction in cell viability at doses of BI 2536 that are highly toxic to MM cells. Cell death proceeds by activation of caspase-8, suggesting that the extrinsic pathway of apoptosis is involved in death of MM cells [19]. Importantly, we observed enhanced toxicity when BI 2536 was combined with either bortezomib or dexamethasone, suggesting synergy between these different classes of drugs.

One of the particular difficulties regarding treatment of MM is overcoming the influence of the bone marrow microenvironment, which is recognized as being of great importance in the pathology of myeloma through niche influences [9,20,21]. In these studies, we show that BI 2536 is able to overcome the protective effects of this microenvironment. The inhibitory effect of BI 2536 is not abrogated when cells are cultured on matrix molecules, laminin, fibronectin, ECM, or, indeed, on bone marrow stromal cells. Thus, BI 2536 can overcome cell-adhesion-mediated drug resistance of MM cells. Furthermore, this drug was able to induce cell death

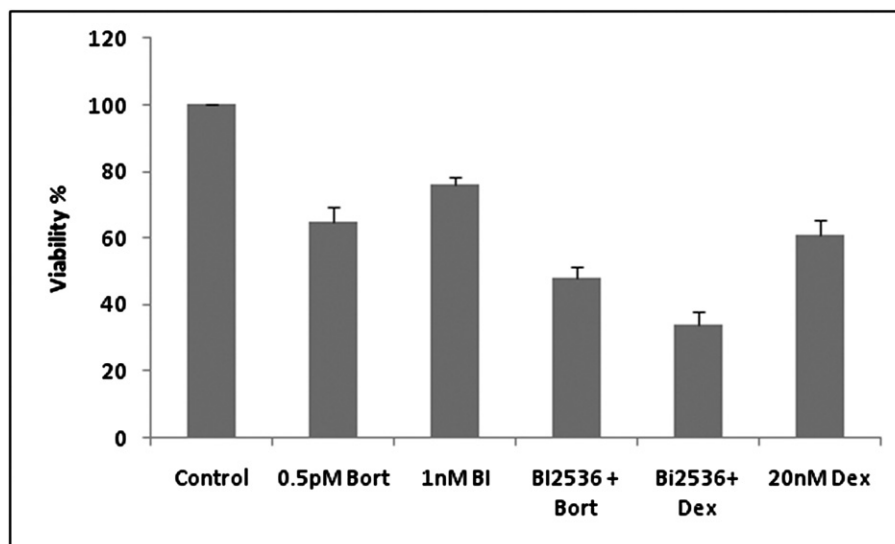


Figure 5. BI2526 enhances the action of bortezomib or dexamethasone. MM.1S were plated at a density of 50,000 cells per well in 96-well plates then treated with 1 nM BI 2536 alone or in combination with 15 nM dexamethasone or 0.5 pM bortezomib for 48 hours. Cell viability was assessed using a WST-1 assay.

in the presence of the potent myeloma cell growth factor, IL-6 [22], and when IL-6 and ECM were used in combination. The combination of ECM with IL-6 represents an environment more akin to that encountered by MM cells *in vivo* than either factor alone. The observation that cell death induced by BI 2536 occurs even when cells are cultured on bone marrow stromal cells also supports these conclusions. These effects are comparable to those seen for the aurora kinase inhibitor VE-465 on MM cells, which is similarly able to overcome the protective effects of both IL-6 and stromal cell adherence [23], although in this study the effects of cell-mediated drug resistance in combination with cytokine effects were not tested.

Interestingly, there is some overlap in the mechanisms of action of these two classes of drugs as aurora kinases are also involved in cell division and Plk1 is activated by aurora A [24]. However, both kinases have different effects on the mitotic process [18]. Numerous inhibitors have been developed against both classes of kinases and some aurora kinase inhibitors have been shown to have effects on multiple myeloma [25–31]. Indeed, ENMD-2076 is currently undergoing phase I trial [18]. Whether Plk1 inhibitors or aurora kinase inhibitors will ultimately prove most useful in the treatment of MM will depend on the efficacy with which they kill tumor cells and the levels of patient tolerance to the drug [32].

In summary, inhibition of Plk1 may have potential use as a therapeutic strategy in MM, either used alone or in combination with other antimyeloma therapies. Our data support translational clinical studies of BI 2536 in myeloma patients, particularly those with relapsed disease.

Acknowledgments

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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