BIRB 796 enhances cytotoxicity triggered by bortezomib, heat shock protein (Hsp) 90 inhibitor, and dexamethasone via inhibition of p38 mitogen-activated protein kinase/Hsp27 pathway in multiple myeloma cell lines and inhibits paracrine tumour growth

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Summary
We have previously shown that heat shock protein (Hsp) 27 or its upstream activator p38 mitogen-activated protein kinase (MAPK) confers resistance to bortezomib and dexamethasone (Dex) in multiple myeloma (MM) cells. This study examined anti-MM activity of a novel p38 MAPK inhibitor, BIRB 796, alone and in combination with conventional and novel therapeutic agents. BIRB 796 blocked baseline and bortezomib-triggered upregulation of p38 MAPK and Hsp27 phosphorylation, thereby enhancing cytotoxicity and caspase activation. The Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) upregulated protein expression and phosphorylation of Hsp27; conversely, BIRB 796 inhibited this phosphorylation and enhanced 17-AAG-induced cytotoxicity. Importantly, BIRB 796 inhibited Hsp27 phosphorylation induced by 17-AAG plus bortezomib, thereby enhancing cytotoxicity. In bone marrow stromal cells (BMSC), BIRB 796 inhibited phosphorylation of p38 MAPK and secretion of interleukin-6 (IL-6) and vascular endothelial growth factor triggered by either tumour necrosis factor-α or tumour growth factor-β1. BIRB 796 also inhibited IL-6 secretion induced in BMSCs by adherence to MM cells, thereby inhibiting tumour cell proliferation. These studies therefore suggest that BIRB 796 overcomes drug-resistance in the BM microenvironment, providing the framework for clinical trials of a p38 MAPK inhibitor, alone and in combination with bortezomib, Hsp90 inhibitor, or Dex, to improve patient outcome in MM.

Keywords: multiple myeloma, p38 mitogen-activated protein kinase, BIRB 796, heat shock protein, bone marrow microenvironment.
mitochondrial release of second mitochondria-derived activator of caspases (Smac) and thereby confers resistance in MM cells (Chauhan et al., 2003b). p38 MAPK regulates Hsp27 through either MAPK-activated protein kinase (MAPKAPK)-2 and/or MAPKAPK-5 activation (Landry & Huot, 1995; New et al., 1998; de Graauw et al., 2005), and we have demonstrated that the p38 MAPK inhibitor SCIO-469 sensitises MM cells to bortezomib by either downregulating or inactivating Hsp27 (Hideshima et al., 2004a).

The present study attempted to establish and validate a strategy to enhance the drug efficacy of bortezomib and Dex by using the p38 MAPK inhibitor, BIRB 796. Although BIRB 796 alone did not induce significant growth inhibition, it augmented cytotoxicity triggered by bortezomib, the Hsp90 inhibitor 17-allylamino-17-demethoxy-geldanamycin (17-AAG), or Dex, associated with inhibition of Hsp27 phosphorylation. In BMSCs, BIRB 796 inhibited secretion of IL-6 and VEGF triggered by either tumour necrosis factor (TNF)-α or tumour growth factor (TGF)-β1. Importantly, BIRB 796 also inhibited IL-6 production and paracrine MM cell growth in the context of BMSCs. These studies therefore suggest that BIRB 796 overcomes growth and drug resistance in the BM microenvironment, providing the framework for clinical trials of a p38 MAPK inhibitor, alone or combined with bortezomib, Hsp90 inhibitor and Dex, to improve patient outcome in MM.

Materials and methods

Reagents

Recombinant human TNF-α and TGF-β1 were obtained from R&D systems (Minneapolis, MN, USA). Bortezomib was obtained from Millennium Pharmaceuticals (Cambridge, MA, USA), diluted in dimethyl sulphoxide (DMSO; 1 mmol/l of stock solution), and kept at −20°C until use. Dex was purchased from Sigma Chemical Co. (St Louis, MO, USA). The Hsp90 inhibitor 17-AAG was purchased from Calbiochem (San Diego, CA, USA).

The p38 MAPK inhibitor, BIRB 796, was provided by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, CT, USA), dissolved in DMSO, and stored at −80°C until use. BIRB 796 is a highly potent inhibitor of p38α and β, at low nmol/l concentrations in kinase assays (Pargellis et al., 2002; Regan et al., 2002; Kuma et al., 2005). In an in vitro cell culture system, BIRB 796 inhibited TNF-α production in THP.1 cells at a 50% inhibitory concentration (IC₅₀) of 18 nmol/l, as well as in human whole blood at an IC₅₀ of 780 nmol/l. Plasma levels of BIRB 796 in humans after oral ingestion of 50 or 600 mg BIRB 796 at 3·5–5·h were 0·74 ± 0·25 μmol/l or 7·38 ± 1·64 μmol/l respectively (Branger et al., 2002).

MM-derived cell lines

The cells MM.1S and MM.1R were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL, USA). U266 and RPMI8226 human MM cells were obtained from American Type Culture Collection (Rockville, MD, USA). OPM1 MM cells were kindly provided by Dr Edward Thompson (University of Texas Branch, Galveston, TX, USA). RPMI-Dox40 cells were kindly provided by Dr William Dalton (Lee Moffitt Cancer Center, Tampa, FL, USA). All cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Sigma Chemical Co.), 2 μmol/l of l-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY, USA).

Primary MM Cells and BMSCs from MM Patients

Tumour cells (>90% CD138⁺) freshly isolated from MM patients were purified by CD138 positive selection using CD138 (Syndecan-1) Micro Beads and the auto manual ability classification system (MACS) magnetic cell sorter (Miltenyi Biotec Inc., Auburn, CA, USA). BM mononuclear cells separated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA) were cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma Chemical) supplemented with 20% heat-inactivated FBS, 2 μmol/l l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin for 3–6 weeks to generate BMSCs. Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients in accordance with the Helsinki protocol.

Growth inhibition assay

The growth inhibitory effect of BIRB 796 on MM cell line growth was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT, Sigma Chemical) dye absorbance, as previously described (Yasui et al., 2005a).

Cell cycle analysis

The MM.1S cells were treated with either 400 nmol/l BIRB 796 or DMSO control 24 h prior to culture with 1 μmol/l Dex for an additional 24 h, harvested, washed with phosphate-buffered saline, fixed with 70% ethanol, and treated with 5 μg/ml of RNase (Roche Diagnostics Corp., Indianapolis, IN, USA). Cells were then stained with propidium iodide (PI; Sigma Chemical; 10 μg/ml), and the cell cycle profile was analysed on RXP Cytomics software on an Epics flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA) as in our prior studies (Yasui et al., 2005a).

Effect of BIRB 796 on MM cells adherence to BMSCs

To evaluate the effect of drug on BMSCs and on growth of MM cells adherent to BMSCs, BMSCs (1·0 × 10⁶ cells/well) were cultured with or without BIRB 796 for 12 h in 96-well plates (Costar, Cambridge, MA, USA); and then cultured with...
or without MM.1S cells for a further 48 h. DNA synthesis was measured by \[^{3}H\]-thymidine (Perkin Elmer, Boston, MA, USA) uptake, as described previously (Yasui et al, 2005b). Culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA; DuoSet, R&D Systems) to evaluate IL-6 and VEGF concentrations, as previously described (Hideshima et al, 2001a). All experiments were performed in triplicate.

**Immunoblotting**

MM.1S cells were treated with BIRB 796 in the presence or absence of bortezomib, Dex, or 17-AAG; cells were harvested, washed and lysed, as in prior studies (Yasui et al, 2005b). Cell lysates were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to polyvinylidenedifluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA), and immunoblotted with anti-phospho-p38 MAPK, p38 MAPK, phospho-Hsp27, Hsp27, caspase-8, caspase-9 and poly adenosine diphosphate ribose polymerase (PARP) (Cell signalling, Beverly, MA, USA); as well as with anti-actin and α-tubulin Abs (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Statistical analysis**

Statistical significance of differences observed in drug-treated versus control cultures was determined using a Student’s t-test. The minimal level of significance was *P* < 0.01.

**Results**

**BIRB 796 inhibits phosphorylation of p38 MAPK in MM cells**

We first examined the effect of BIRB 796 on baseline phosphorylation of p38 MAPK and Hsp27 in MM cell lines. BIRB 796 (25–400 nmol/l) treatment for 2 h strongly inhibited phosphorylation of both p38 MAPK and Hsp27 in MM.1S cells (Fig 1A). Although Hsp27 was highly expressed in U266, RPMI8226 and OPM1 cell lines (Fig S1A), BIRB 796 (25–400 nmol/l) treatment also inhibited phosphorylation of both p38 MAPK and Hsp27 in these cells (Fig 1A and B). However, 800 nmol/l of BIRB 796 as a single agent did not trigger significant growth inhibition at 72 h, as assessed by MTT assay (Fig S1B). BIRB 796 has been reported as a long-acting p38 MAPK inhibitor (Pargellis et al, 2002) that may contribute to the inhibition of p38 MAPK phosphorylation even at 48-h treatment (data not shown).

**BIRB 796 enhances bortezomib-induced cytotoxicity in MM cells**

We next examined the inhibitory effect of BIRB 796 on phosphorylation of both p38 MAPK and Hsp27 in combination with bortezomib in MM.1S cells. Consistent with our previous studies (Mitsiades et al, 2002), bortezomib significantly upregulated Hsp27 expression and phosphorylation. Importantly, pretreatment with BIRB 796 inhibited upregulation of phosphorylation and protein expression of Hsp27 in MM.1S cells (Fig 2A). We next examined whether BIRB 796 augmented MM cytotoxicity triggered by bortezomib. As shown in Fig 2B and C, BIRB 796 significantly enhanced bortezomib-triggered cytotoxicity in MM.1S cells and RPMI8226 cells, in a dose-dependent manner. We then examined the mechanism whereby inhibition of p38 MAPK augments cytotoxicity of bortezomib. BIRB 796 enhanced cleavage of caspase-8, caspase-9, and PARP triggered by bortezomib in MM.1S cells (Fig 2D). Moreover, the pan-caspase inhibitor Z-VAD-FMK abrogated enhancement of bortezomib cytotoxicity by BIRB796 (Fig S1C), suggesting that BIRB796 enhanced bortezomib-induced apoptosis in MM cells. These results are consistent with our previous studies, which demonstrated that bortezomib activated p38 MAPK followed by upregulation of phosphorylation and phosphorylation of Hsp27, which is inhibited by p38 MAPK inhibitors (Hideshima et al, 2004a).

**BIRB 796 with Hsp90 inhibitor 17-AAG enhances bortezomib-induced cytotoxicity**

Heat shock protein 90 is a molecular chaperone upregulated by many agents that trigger cell stress and/or apoptosis. Specifically, it was demonstrated that the Hsp90 inhibitor 17-AAG enhanced bortezomib-triggered cytotoxicity in MM cells...
We therefore examined whether BIRB 796 could further augment cytotoxicity induced by bortezomib plus 17-AAG. Interestingly, Hsp90 inhibition using 17-AAG significantly induced Hsp27 expression and phosphorylation at 12 h in MM.1S cells as well as in patient MM cells, which was inhibited by BIRB 796 (Fig 3A and B). On the other hand, inhibition of Hsp27 using BIRB 796 did not induce Hsp90 expression (Fig S1D). At 72 h, BIRB 796 modestly enhanced cytotoxicity triggered by 17-AAG in MM.1S cells (Fig 3C) as well as in patient MM cells (Fig 3D). 17-AAG enhanced phosphorylation of Hsp27 triggered by bortezomib, which was blocked by BIRB 796 (Fig 3E). Importantly, BIRB 796 significantly increased an enhancement of bortezomib cytotoxicity by 17-AAG at 24 h (Fig 3F). These results suggest that BIRB 796 inhibited induction of Hsp27 phosphorylation by 17-AAG, thereby increasing enhancement of bortezomib cytotoxicity by 17-AAG in MM.

**BIRB 796 sensitises MM cells to Dex**

Chauhan et al (2003b) showed that blockade of Hsp27 restored sensitivity to Dex-induced apoptosis in MM cells, indicating that Hsp27 confers Dex resistance in MM cells. As shown in Fig 4A, BIRB 796 inhibited baseline and Dex-induced phosphorylation of both p38 MAPK and Hsp27 in MM.1S cells. We next examined whether BIRB 796 could augment cytotoxicity triggered by Dex in MM.1S cells. Although MM.1S cell proliferation was strongly inhibited by Dex alone at 24–72 h, BIRB 796 significantly enhanced its growth inhibition (Fig 4B). Cell cycle profiling suggests that BIRB 796 augmented Dex-mediated growth inhibition by enhancing apoptosis (Sub-G1 portion: control = 6.1%, BIRB 796 alone = 8.0%, Dex alone = 34.7%, BIRB 796 plus Dex = 45.7%) (Fig 4C).

**BIRB 796 downregulates IL-6 and VEGF secretion in BMSCs triggered by TNF-α and TGF-β1**

As we have shown that p38 MAPK inhibitors VX-745 and SCIO-469 inhibit IL-6 and VEGF secretion from BMSCs of MM patients (Hideshima et al, 2003, 2004a), we similarly examined whether BIRB 796 could inhibit secretion of these cytokines from BMSCs. As in MM cells, BIRB 796 also inhibited p38 MAPK phosphorylation in patient BMSCs (Fig 5A). BIRB 796 significantly inhibited baseline secretion of IL-6 and VEGF in BMSCs. Both TNF-α and TGF-β1 are potent inducers of IL-6 and VEGF secretion in BMSCs (Hideshima et al, 2001b; Hayashi et al, 2004); we therefore investigated whether BIRB 796 could also inhibit IL-6 and VEGF secretion triggered by these cytokines. Although TNF-α and TGF-β1 upregulated p38 MAPK phosphorylation, BIRB 796 abrogated this upregulation (Fig 5B), associated with downregulation of IL-6 and VEGF.
Therefore, secretion of IL-6 and VEGF in BMSCs induced by TNF-α and TGF-β1 was significantly inhibited by BIRB 796 (Fig 5C and D).

**BIRB 796 inhibits MM cell growth triggered by adherence to BMSCs**

Multiple myeloma cell adherence to BMSCs is known to increase IL-6 secretion in BMSCs and triggers MM cell growth, we next therefore examined whether BIRB 796 inhibited IL-6 secretion and growth of MM cell adherent to BMSCs. BIRB 796 significantly abrogated IL-6 secretion and paracrine MM cell growth in the presence of BMSCs (Fig 5E and F). These results suggest that BIRB 796 downregulates IL-6 secretion and inhibits MM cell growth in the BM microenvironment.

**Discussion**

The p38 MAPKs were initially identified as mammalian homologues of the yeast protein Hog1 that senses osmolarity change (Han et al, 1994). At least four isoforms of p38 MAPK are defined: p38 MAPK α, β, γ and δ (Kyriakis & Avruch, 2001). p38 MAPKs are phosphorylated and activated at threonine and tyrosine by dual kinases MKK3 and MKK6. p38 MAPKs regulate the function of transcription factors, kinases, or phosphatases such as activating transcription factor 2 (ATF-2), mads box transcription enhancer factor 2 (MEF2), MAPKAPK or cell division cycle 25 (CDC25) (Bulavin et al, 2001; Johnson & Lapadat, 2002). Moreover, p38 MAPK, regulating Hsp27 through either MAPKAPK-2 or p38 MAPK signalling pathway, is therefore involved in a variety of cellular responses.

The involvement of p38-MAPK in apoptosis varies in different cell systems. It has been shown that p38-MAPK signalling can promote cell death (Takekawa et al, 2000; Porras et al, 2004), whereas it can also enhance survival (Park et al, 2002) and cell growth (Bulavin et al, 2001; Juretic et al, 2001). Given that the p38-MAPK pathway mediates apoptosis or survival depending on the cancer cell type (Wada & Penninger, 2004), pharmacological inhibition of p38 MAPK could...
represent targeted treatment in certain cancers. For example, pharmacological inhibition of p38 MAPK inhibitor using SB202190 induced apoptosis in T-cell leukaemia Jurkat cells (Nemoto et al., 1998). Moreover, in acute promyelocytic leukaemia cells, either all-trans retinoic acid (ATRA) (Alsayed et al., 2001) or arsenic trioxide (Verma et al., 2002) activated p38 MAPK and its downstream effector MAPKAPK-2. Inhibition of p38 MAPK using SB203580 enhanced growth inhibition triggered by ATRA or arsenic trioxide. In both cases, pharmacological inhibition of p38 MAPK promoted the induction of anti-leukaemic responses, suggesting common downstream regulatory mechanisms (Platanias, 2003).

The present study demonstrated that pharmacological inhibition of p38 MAPK using a novel p38 MAPK inhibitor BIRB 796 inhibited phosphorylation of p38 MAPK in MM cells at clinically achievable nmol/l concentrations. BIRB 796 alone had marginal growth inhibitory effect, suggesting that inhibition of p38 MAPK alone does not inhibit growth-signalling cascades in MM cells. Importantly, BIRB 796 blocked baseline and bortezomib-triggered upregulation of p38 MAPK and Hsp27 phosphorylation, associated with enhanced cytotoxicity, caspase-8/caspase-9 activation, and PARP cleavage. These data are consistent with our previous study using a different p38 MAPK inhibitor SCIO-469 (Hideshima et al., 2004a).

We next examined the combination with Hsp90 inhibitor 17-AAG, a geldanamycin analogue that inhibits chaperone function of Hsp90 and has pleiotropic proapoptotic effects in MM cells, including sensitising them to other anticancer agents such as bortezomib (Mitsiades et al., 2006). Interestingly, 17-AAG upregulated protein expression and phosphorylation of Hsp27 probably as a stress response; conversely, BIRB 796 inhibited this phosphorylation. Moreover, BIRB 796 modestly enhanced 17-AAG-induced cytotoxicity. We have already demonstrated that bortezomib induced Hsp’s, including Hsp27, Hsp70 and Hsp90, in MM cells (Mitsiades et al., 2002, 2006); and Hsp’s inhibit apoptotic signalling at several levels (Jolly & Morimoto, 2000; Xanthoudakis & Nicholson, 2000; Concannon et al., 2003), pharmacological inhibition of Hsp’s in bortezomib-treated MM cells might enhance apoptosis (Mitsiades et al., 2006). Importantly, BIRB 796 enhanced the cytotoxicity induced by 17-AAG plus bortezomib. These preclinical studies suggest that BIRB 796 is a potent combination with bortezomib and 17-AAG.

Our previous studies demonstrated that Hsp27 overexpression was associated with resistance to Dex in MM; conversely, Hsp27 antisense restored sensitivity to Dex via triggering release of Smac, followed by an activation of caspase-9 and caspase-3 (Chauhan et al., 2003b). We next investigated whether pharmacological inhibition of Hsp27 using p38 MAPK inhibitor enhanced the cytotoxicity triggered by Dex. Importantly, BIRB 796 also augmented cytotoxicity of Dex in MM cells, attendant with inhibition of Hsp27 phosphorylation.
Hsp27 belongs to the family of small Hsp’s that are ubiquitously expressed and exhibit the capacity to form oligomeric structures (Arrigo & Pauli, 1988; Mehlen & Arrigo, 1994). Diverse agents, such as TNF-α and oxidative stress, have been reported to be potent inducers of phosphorylation of Hsp27. Overexpression of Hsp27 has been shown to protect cells against cell death induced by TNF-α, staurosporine, anti-Fas antibody or oxidative stress (Mehlen et al, 1995, 1996a; Mehlen et al, 1996b). Hsp27 also acts as a molecular chaperone that can interfere with caspase-3 activation and cytochrome c release, thereby inhibiting apoptosis (Bruey et al, 2000; Pandey et al, 2000). Recently, de Graauw et al (2005) demonstrated that p38 MAPK activation results in a rapid phosphorylation of Hsp27, thus suppressing apoptosis. Although the signalling mechanisms involved in the anti-apoptotic function of Hsp27 are not fully defined, these studies indicate that Hsp27 has an anti-apoptotic potential, and that inhibition of Hsp27 to enhance apoptosis triggered by novel agents is a promising therapeutic strategy.

Accumulating evidence suggests that the tumour microenvironment plays an important role in tumourigenesis and tumour progression (Mueller & Fusenig, 2004). In MM, the BM microenvironment includes immune cells [including T cells, macrophages, natural killer (NK) cells, and dendritic cells (DCs)]; BMSCs; BM endothelial cells; cells involved in bone homeostasis (including osteoclasts and osteoblasts); as well as extra cellular matrix (ECM) proteins and secreted growth factors conferring paracrine MM growth and drug resistance (Hideshima & Anderson, 2002; Hideshima et al, 2004b; Mitsiades et al, 2004). We have already shown that the p38 MAPK inhibitor VX-745 inhibited paracrine MM cell growth, associated with downregulation of IL-6 secretion in BMSCs (Hideshima et al, 2003). The present study demonstrated that BIRB 796 significantly inhibited constitutive and TNF-α-triggered IL-6 and VEGF secretion from BMSCs, consistent with our previous study (Hideshima et al, 2003). BIRB 796 also inhibited IL-6 and VEGF secretion in BMSCs triggered by TGF-β1, which modulates production of IL-6 and VEGF.

Fig 5. BIRB 796 inhibits baseline phosphorylation of p38 mitogen-activated protein kinase (MAPK) and phosphorylation induced by tumour necrosis factor (TNF)-α and by tumour growth factor (TGF)-β1, as well as paracrine multiple myeloma (MM) cell growth. (A) MM patient bone marrow stromal cells (BMSCs) were cultured in the presence or absence of BIRB 796 (25, 100 and 400 nmol/l) for 2 h. (B) BMSCs were cultured with control media or 400 nmol/l of BIRB 796 for 2 h and then with TNF-α (5 ng/ml) or TNF-β1 (5 ng/ml) for an additional 10 min. Total cell lysates were subjected to immunoblotting using anti-phospho-p38 MAPK (p-p38) and p38 MAPK (p38) antibodies. (C, D) BMSCs were cultured with TNF-α (5 ng/ml) or TNF-β1 (5 ng/ml) in the presence or absence of BIRB 796 (25, 100, or 400 nmol/l) for 48 h. Interleukin-6 (IL-6) (C) and vascular endothelial growth factor (D) in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA); data represent mean (±SD) of triplicate cultures. *P < 0.01. (E) 1 × 10⁵ BMSCs were seeded in 96-well plates for 12 h; cultured with control media or with BIRB 796 (25, 100 and 400 nmol/l, 12 h); and then with 2 × 10⁵ MM.1S cells/well for an additional 48 h. IL-6 concentration in supernatants was analysed by ELISA; data represent mean (±SD) of triplicate cultures. *P < 0.01. DNA synthesis was evaluated by thymidine uptake assay; data represent mean (±SD) of triplicate cultures. *P < 0.01.
thereby conferring MM cell growth in the BM microenvironment (Hayashi et al., 2004; Yasui et al., 2006). Moreover, BIRB 796 inhibited IL-6 production in BMSCs triggered by adherence of MM cells as well as paracrine MM cell growth. These results suggest that BIRB 796 inhibits cytokine (TNF-α, TGF-β1, IL-6, VEGF)-induced paracrine MM cell growth in the BM microenvironment. In MM bone disease, Nguyen et al. (2006) demonstrated that pharmacological inhibition of p38 MAPK using SCIO-469 inhibits: induction of osteoclast-activating factors including RANKL, IL-11 and MIP-1α in BMSCs triggered by TGF-β1; RANKL-induced p38MAPK activation in human osteoclast precursors; and prevents osteoclast formation in vitro. Another possibility of p38 MAPK inhibitor is restoration of immune deficiency in MM. p38 MAPK activation, triggered by myeloma cell conditioning medium inhibited BM-derived DCs function, evidenced by the downregulated expression of DC-related surface molecules, decreased IL-12, and compromised capacity of the cells to activate allospecific T cells; conversely, pharmacological inhibition of p38 MAPK using SB205890 restored the phenotype, cytokine secretion and function of MM cell conditioning medium-treated BMDCs (Wang et al., 2006). Further investigation is needed to describe the role of p38 MAPK in the BM microenvironment, as well as in bone lysis and immunosuppression, in MM.

In summary, although pharmacological inhibition of p38 MAPK using BIRB 796 triggered a marginal growth inhibitory effect, it enhanced MM cytotoxicity of bortezomib, 17-AAG and Dex, associated with inhibition of Hsp27 phosphorylation. BIRB 796 inhibited Hsp27 phosphorylation induced by 17-AAG plus bortezomib, thereby enhancing cytotoxicity. In BMSCs, BIRB 796 inhibited phosphorylation of p38 MAPK and secretion of IL-6 and VEGF triggered by either TNF-α or TGF-β1. BIRB 796 also inhibited IL-6 production in BMSCs triggered by adherence of MM cells, thereby inhibiting MM cell proliferation. Our results therefore provide the preclinical rationale for clinical trials of a p38 MAPK inhibitor, BIRB 796, alone and in combination with Hsp90 inhibitor, bortezomib, or Dex, to enhance drug sensitivity and improve patient outcome in MM.

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References


**Supplementary material**

The following supplementary material is available for this article online:

**Fig S1A.** Expression and phosphorylation of heat shock protein 27 in multiple myeloma cells.

**Fig S1B.** BIRB 796 has minimum effect on multiple myeloma cell viability.

**Fig S1C.** MM.1S cells were cultured with 400 nmol/l of BIRB 796 for 12 h; 25 μmol/l of Z-VAD (Z-VAD-FMK: Calbiochem, San Diego, CA, USA) was then added for an additional 1 h, followed by control media (clear bars) and 5 (grey bars) or 10 nmol/l (black bars) of bortezomib for the last 24 h. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide assays, and data represent mean (± SD) of quadruplicate cultures.

**Figure S1D.** BIRB 796 does not change expression levels of heat shock protein 90. MM.1S cells were cultured with control media or 400 nmol/l of BIRB796 for 24 h.

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