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Targeting executioner procaspase-3 with the procaspase activating compound B-PAC-1 induces apoptosis in multiple myeloma cells

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Abstract

Multiple myeloma (MM) is a plasma cell neoplasm that has a low apoptotic index. We investigated a new class of small molecules that target the terminal apoptosis pathway, called procaspase activating compounds (PACs), in myeloma cells. PAC agents (PAC-1 and B-PAC-1) convert executioner procaspases (procaspase-3, -6 and -7) to active caspase-3, -6, and -7, which cleave target substrates to induce cellular apoptosis cascade. We hypothesized that targeting this terminal step will overcome survival and drug-resistance signals in myeloma cells and induce programmed cell death. Myeloma cells expressed executioner caspases. In concert, our studies demonstrated that B-PAC-1 is cytotoxic to chemotherapy resistant or sensitive myeloma cell lines (n=7) and primary patient cells (n=11). Exogenous zinc abrogated B-PAC-1-induced cell demise. B-PAC-1-treatment-induced apoptosis was similar in the presence or absence of growth-promoting cytokines such as interleukin-6 and hepatocyte growth factor. Presence or absence of anti-apoptotic proteins such as BCL-2, BCL-X_L, or MCL-1 did not impact B-PAC-1-mediated programmed cell death. Collectively, our data demonstrate the proapoptotic effect of B-PAC-1 in MM and suggests that activating terminal executioner procaspase-3, -6 and -7 bypasses survival and drug-resistance signals in myeloma cells. This novel strategy has the potential to be an effective anti-myeloma therapy.

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Conflicts of interest

VG received Sponsored Research Agreement from Vanquish Oncology. All other authors declare no financial competing interests.

Keywords

procaspase activating compound; procaspase-3; multiple myeloma; B-PAC-1; executioner procaspase

Introduction

Multiple myeloma (MM) is currently the second most common hematological malignancy in the US, and accounts for approximately 10% of all hematologic cancers [1, 2]. In MM, the excess plasma cells in the bone marrow interfere with the production of normal blood cells and lead to devastating sequelae, including osteolytic bone lesions, renal disease, and immunodeficiency [3]. Currently, the most common treatment administered to myeloma patients include high dose chemotherapy of dexamethasone, bortezomib, and lenalidomide followed by autologous hematopoietic stem-cell transplantation [4]. However, these treatments are not curative and most patients eventually acquire resistance to these chemotherapeutic agents and relapse.

A main distinction of cancer is its resistance to natural apoptotic signals [5], enabling cancerous cells to survive and divide even in the presence of endogenous proapoptotic stimuli [6]. These signals result from either up or down regulation of key proteins in the apoptotic cascade that can occur in the intrinsic and extrinsic apoptotic pathways [5, 7, 8]. The intrinsic pathway is activated by cellular stress such as DNA damaging agents, leading to the release of cytochrome c from the mitochondria. Cytochrome c forms a complex with APAF1 and initiator caspase-9 called the apoptosome that converts the executioner procaspases-3, -6, and -7 to active caspase-3, -6 and -7. These active caspases target numerous substrates for cleavage, eventually leading to apoptosis. Similarly, the extrinsic pathway is activated upon stimulation of the tumor necrosis factor family death receptors, which convert procaspase-8 to caspase-8 and activates the executioner procaspases leading to apoptosis.

Myeloma cells have a low apoptotic index which decreases as the disease progresses from monoclonal gammopathy of undetermined significance (MGUS) to smoldering or indolent myeloma (SMM/IMM) to newly diagnosed MM [9]. This decrease in the apoptotic index is often due to overexpression of survival proteins including the BCL-2 family proteins, BCL-2, BCL-X_L and MCL-1 [10–14], and the IAP family proteins, cIAP1, cIAP2 and XIAP [15–17]. The deregulation of these survival proteins prevents proapoptotic signals from being transmitted to activate executioner caspases and, thus, cause cancerous cells to proliferate uncontrollably [18].

The proteolytic conversion of procaspase-3 (-6 or -7) to caspase-3 (-6 or -7) results in the active ‘executioner’ caspase that subsequently catalyzes the hydrolysis of many protein substrates [18]. Active caspase-3 is a homodimer of heterodimers and is typically activated through the action of caspase-8 and caspase-9 [18]. To prevent premature activation of procaspase-3, the zymogen utilizes a tripartic acid ‘safety catch.’

Procaspase-3 has been found to have elevated concentrations in cells from various cancerous tissues including certain neuroblastomas, lymphomas, leukemias, melanomas and liver cancers [18]. This allows for a therapeutic index to be achieved for caspase-3-directed agents. Due to the focal role of active caspase-3 in successfully inducing apoptosis, the use of a small molecule to directly activate procaspase-3 has been suggested as a tactical approach in targeted cancer therapy. Such an effort would bypass the damaged apoptotic cascade in cancer cells and directly convert procaspase-3 to caspase-3 [6].

Procaspase activating compound 1 (PAC-1) and its new generation analog (B-PAC-1) are a class of small molecules that stimulate the conversion of procaspase-3 to caspase-3 [6]. Mechanistically, PAC-1 induces activation of procaspase-3 *in vitro* via sequestration of inhibitory zinc ions. Evidences have shown that zinc binding is critical to the ability of PAC-1 to induce death in cancer cells [6]. PAC-1 induces the autoactivation of caspase-3 and caspase-3-mediated cleavage of anti-apoptotic proteins (such as BCL-2 and BCL-X_L), which in turn may induce depolarization of the mitochondrial membrane and amplify the apoptotic effect. PAC-1 has entered Phase I trials and B-PAC-1 is being evaluated to move to clinic.

Procaspase-3 presents itself as a strategic therapeutic target capable of bypassing upstream mutational inactivation of proapoptotic proteins. Described herein are experiments testing the effectiveness and mechanism of action of B-PAC-1, a new investigational drug in multiple myeloma cells.

Materials and methods

Cell cultures and reagents

All cell lines were maintained in a 37 °C humidified incubator with 5% CO₂. Myeloma cell lines were grown in media as indicated in Table 1 [19–23]. HL-60/Neo, HL-60/BCL-2 and HL-60/BCL-X_L cell lines were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. Mouse embryo fibroblasts that were wild type for MCL-1 (WT MCL-1) or deleted for MCL-1 (MCL-1^{-/-}) were maintained in DMEM media with no glucose and was supplemented with 1× MEM non-essential amino acid (Gibco, Grand Island, NY), 1× penicillin/streptomycin, 0.2 mM β-mercaptoethanol (Sigma, St Louis, MO), 10% fetal bovine serum and 2mM L-glutamine. All cell lines were authenticated and tested for *Mycoplasma* contamination by the UT MD Anderson Cancer Center Characterized Cell Line Core. The procaspase-3 activating compounds (PAC-1, B-PAC-1 (previously known as L14R8) and PAC-1a) were a kind gift from Dr. Hergenrother (University of Illinois at Urbana-Champaign, IL).

Cell apoptosis assays

Cells were harvested after treatment with PAC agents for 24 hours. Cells were centrifuged at 1500 rpm for 5 min, washed once with 1× phosphate buffered saline and incubated with annexin V-FITC (Pharmingen Biosciences, San Diego, CA) for 10 min. Cells were then treated with 0.5 µg/ml propidium iodide (PI) and analyzed on a BD FACSCalibur (BD Biosciences, San Jose, CA).

Co-culture of U266 cells with NKtert cells

U266 cells were co-cultured with NKtert stromal cells at a ratio of 20:1. NKtert cells were seeded in 12-well plates at a concentration of 2.5×10^4 cells/ml. After 14–16 hours, U266 cells were seeded on NKtert cells at a concentration of 5×10^5 cells/ml. Co-cultured cells were incubated for 2 hours before the treatments were added. After 24 hours, U266 cells, which are suspension cells, were removed for cell apoptosis analysis. NKtert cells were also analyzed for cell apoptosis after detaching them with Accutase Cell Detachment Solutions (Innovative Cell Technologies, San Diego, CA).

Immunoblot analysis

Whole-cell lysates were prepared using $1 \times$ RIPA buffer (Millipore, Billerica, MA), supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN). Cells were sonicated in RIPA buffer for 2×3 min and centrifuged at 4°C for 10 min at 14,000 rpm. The supernatant was used for further analysis. Equal concentration of lysates was separated on 4–12% Criterion XT Bis-Tris precast gels (Bio-Rad, Hercules, CA).

Antibodies used in immunoblot analysis were caspase-3, -6 and -7 (Cell Signaling Technologies, Danvers, MA), cIAP2 (Epitomics, Burlingame, CA), MCL-1 (Santa Cruz Biotechnology, Dallas, TX), cIAP1 and GAPDH (Abcam, Cambridge, MA), cleaved PARP and XIAP (BD Biosciences, San Jose, CA). Immunoblots were scanned on an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE) and quantitated using Image Studio Lite Ver 3.1 (LI-COR Biosciences, Lincoln, NE). Caspase positive and negative control cell lysates were obtained from Cell Signaling Technologies.

Cytotoxicity analysis of primary bone marrow CD138+ cells

Bone marrow samples were obtained from patients with myeloma through a protocol approved by the MD Anderson Cancer Center Institutional Review Board (IRB) and in accordance with the Declaration of Helsinki. CD138+ cells were isolated from bone marrow by the institutional myeloma core facility [22]. For some bone marrow samples, cells were received unsorted. For these samples, after drug treatment cells were centrifuged and were labeled with monoclonal CD138 antibody conjugated to APC (Miltenyi Biotec, Auburn, CA). Both CD138+ sorted and unsorted cells were stained with annexin V-FITC and PI and analyzed as described above.

Statistical analysis

Statistical analysis was done using GraphPad Prism (GraphPad software version 6, San Diego, CA). The analyses used are indicated in the figures.

Results

B-PAC-1 induced apoptosis in a dose- and time-dependent manner

The procaspase-activating compounds (PAC), PAC-1 and B-PAC-1 (a potent analog of PAC-1), are small molecules that initiate apoptosis through targeting procaspase-3. PAC-1 is an ortho-hydroxy N-acyl hydrazine while B-PAC-1 is a second generation analog of PAC-1 that contains benzyloxy and di-t-butyl functional groups that increases its lipophilicity

(Supplementary Figure 1). To examine if targeting procaspase-3 will be a viable therapeutic target in multiple myeloma, we first examined procaspase-3 protein expression in 8 multiple myeloma cell lines: RPMI-8226, ANBL-6, ANBL-6/V10R (bortezomib-resistant), KAS-6/1, KAS-6/R10R (lenalidomide-resistant), KAS-6/V10R (bortezomib-resistant), U266 and MM.1S (Fig. 1A). Procaspase-3 protein expression was readily detected at steady-state levels in all the tested cell lines, suggesting that PAC agents might be effective in inducing apoptosis in myeloma cells. In addition to procaspase-3, we also examined the protein expression of the other executioner procaspases, procaspase-6 and procaspase-7. As shown in Figs. 1B and 1C, both procaspases are readily detectable at steady-state levels in myeloma cell lines.

We examined the cytotoxicity of PAC-1 and B-PAC-1 in 2 myeloma cell lines, MM.1S and U266. A dose-response experiment with PAC-1, B-PAC-1 and PAC-1a (a structurally-related, inactive analog of PAC-1 that was used as a negative control) demonstrated that B-PAC-1 increased the percentage of cells stained with annexin V and propidium iodide (PI) in a dose-dependent manner compared to control cells treated with DMSO (Figs. 2A and 2B). Treatment of MM.1S cells with 3 and 10 μ M B-PAC-1 increased annexin V+/PI+ cells by 70% and 93%, respectively (Fig. 2C). In U266 cells, 3 and 10 μ M B-PAC-1 increased apoptotic cells by 39% and 84%, respectively (Fig. 2D). MM.1S cells displayed more sensitivity to B-PAC-1 (IC_{50} of 2.6 μ M) than U266 cells (IC_{50} of 5 μ M). In contrast, neither PAC-1 nor PAC-1a induced apoptosis in these cell lines (Figs. 2 C–D). We, therefore, continued our investigations with B-PAC-1 in myeloma cells to further evaluate its efficacy.

To examine the kinetics of B-PAC-1 action in MM.1S and U266 cells, we conducted a time-course experiment and examined B-PAC-1 cytotoxicity after treatment with DMSO (control cells), 3, and 10 μ M B-PAC-1 for 3, 6 and 24 hours (Figs. 2E and 2F). Both MM.1S and U266 cells demonstrated a time-dependent decrease in cell viability. Consistent with the dose-response experiments, MM.1S cells exhibited greater sensitivity than U266 cells to B-PAC-1. In MM.1S cells, cell viability decreased as early as 3 hours after treatment with 10 μ M B-PAC-1. These data illustrate that B-PAC-1 induced apoptosis in myeloma cell lines in a dose- and time-dependent manner.

B-PAC-1-induced apoptosis is inhibited by zinc

Next, we examined the mechanism of action of B-PAC-1 in myeloma cell lines. Procaspase-3 is inhibited by labile zinc ions and becomes activated when cleaved by caspase-9 at specific aspartic acid residues. Studies have shown that PAC-1 activates procaspase-3 by chelating zinc ions, allowing it to auto-activate [6]. To examine if B-PAC-1 activated procaspase-3 in myeloma cells through chelating zinc ions, MM.1S cells were treated with 3 or 10 μ M B-PAC-1 in the absence or presence of 100 μ M zinc. As shown in Fig. 3A, 3 and 10 μ M B-PAC-1 treatment reduced the viability of MM.1S cells; however, addition of excess exogenous zinc inhibited the function of B-PAC-1 and cell viability was restored to the levels observed in control cells. To examine if zinc-dependent inhibition of apoptosis was specific to B-PAC-1, we treated cells with staurosporine, a natural product isolated from the bacterium *Streptomyces staurosporeus*, that can induce apoptosis. Addition of exogenous zinc did not inhibit staurosporine-mediated cell apoptosis (Fig. 3A), suggesting that staurosporine does not depend on zinc for its cytotoxic activity.

To analyze B-PAC-1-mediated apoptosis at the molecular level, we conducted immunoblot analysis after treatment of MM.1S cells with DMSO (control cells), 5 or 10 μ M B-PAC-1 for 24 hours (Fig. 3B and Supplementary Fig. 2). Compared to DMSO (vehicle) treatment, cells treated with 5 and 10 μ M B-PAC-1 resulted in decrease in the levels of procaspase-3 and increase in the levels of cleaved caspase-3. We used Image Studio Lite Ver 3.1 to semi-quantitatively analyze the immunoblots and normalized the signal to GAPDH (Supplementary Figure 2). The decrease in procaspase-3 levels was associated with an increase in the levels of cleaved PARP, a target of caspase-3, thus confirming the cleaving of procaspase 3 and activation of caspase-3. As in the previous experiment, the addition of 100 μ M exogenous zinc inhibited procaspase-3 cleavage. In contrast, staurosporine treatment increased procaspase-3 cleavage but was not affected by the addition of zinc.

We also examined the effects of B-PAC-1 treatment on the levels of survival proteins, including the IAP proteins (cIAP1, cIAP2 and XIAP) and the BCL-2 family protein MCL-1 (Fig. 3B and Supplementary Fig. 1). B-PAC-1 treatment led to a decrease in the levels of these anti-apoptotic proteins that was reversed by the addition of exogenous zinc, restoring the levels of these proteins to those of control cells.

Since previous studies with PAC-1 demonstrated that it targeted procaspase-7 [6], we examined if B-PAC-1 cleaved the other executioner procaspases, procaspase-7 and procaspase-6, in MM.1S myeloma cells. To examine the effect of B-PAC-1 on procaspase-7, MM.1S cells were treated with DMSO (control cells), 3 or 10 μ M B-PAC-1 for 24 hours. As shown in Fig. 3C (left panel), compared to control-treated cells, 10 μ M B-PAC-1 treatment reduced the levels of procaspase-7 and increased the levels of active caspase-7. To examine if zinc ions inhibited B-PAC-1-mediated cleavage of procaspase-7, MM.1S cells were treated with zinc (control cells), 10 μ M B-PAC-1 or 10 μ M B-PAC-1 in the presence of zinc ions. As shown in Fig. 3C (middle panel), B-PAC-1-mediated cleavage of procaspase-7 was inhibited by the addition of exogenous zinc. Jurkat cells treated with or without staurosporine were run in parallel as positive and negative controls, respectively.

Next, we examined if B-PAC-1 was able to cleave procaspase-6 into active caspase-6. MM.1S cells were treated with DMSO (control cells), 3 or 10 μ M B-PAC-1 for 24 hours. As Fig. 3D shows, compared to control cells, 10 μ M B-PAC-1 decreased the levels of procaspase-6. GAPDH was used as loading control to indicate the difference in expression was not due to differential loading. However, we were unable to detect the active caspase-6 band on the immunoblot. The absence of cleaved caspase-6 could be due to either low levels in myeloma cells or its further degradation. We have observed similar results in the prior published report [24]. Because we were unable to detect cleaved caspase-6 in myeloma cell lines, we used procaspase-6 expression to determine the activity of B-PAC-1 on caspase-6. In summary, these data indicate that in addition to targeting procaspase-3, B-PAC-1 also targeted procaspase-7 and -6 and converted procaspase-7 to active caspase-7, while reducing the levels of procaspase-6.

B-PAC-1 overcame microenvironment-mediated drug resistance

The bone marrow microenvironment supports the growth of myeloma cells and is a source of resistance to anti-myeloma therapeutics due to the release of growth-promoting cytokines

from cells in the bone marrow milieu. To examine if B-PAC-1 can overcome microenvironment-related drug resistance, we investigated the effects of B-PAC-1 in the presence of high levels of the exogenous growth factors interleukin (IL)-6 and hepatocyte growth factor (HGF). MM.1S and U266 cells were treated with 5 μ M B-PAC-1 in the presence of 10 or 50 ng/ml IL-6 (Figs. 4 A–B). Treatment with 5 μ M B-PAC-1 reduced cell viability by >80% in MM.1S cells and >44% in U266 cells. Importantly, B-PAC-1 was able to induce similar levels of apoptosis in the absence and presence of IL-6. Similarly, B-PAC-1 was equally effective in inducing apoptosis in cells grown in the absence or presence of 50 ng/ml HGF (Figs. 4 A–B).

In addition to examining B-PAC-1 cytotoxicity in the presence of exogenous growth factors, we also examined its cytotoxicity when myeloma cells were co-cultured with NKtert cells, a human bone marrow stromal cell line. As shown in Supplementary Figure 3, B-PAC-1 was effective in reducing the viability of U266 cells when cultured alone and also, when co-cultured with NKtert cells, indicating that it is able to overcome the protective bone marrow microenvironment of NKtert cells. However, B-PAC-1 was also toxic to NKtert cells (data not shown).

B-PAC-1 was cytotoxic to drug-resistant myeloma cell lines

Next, we investigated if B-PAC-1 is effective in inducing apoptosis in cells that are resistant to current multiple myeloma therapeutics. FDA-approved drugs for multiple myeloma include dexamethasone, lenalidomide and bortezomib; therefore, we tested cell lines that are sensitive to these agents (MM.1S, KAS-6/1) and cells that are resistant to lenalidomide (MM1/R10R, KAS-6/R10R), dexamethasone (MM.1R) or bortezomib (KAS-6/V10R) for sensitivity to B-PAC-1. A dose-response experiment with B-PAC-1 demonstrated that apoptosis was induced in all of these cell lines (Fig. 5). The similarity in the response to B-PAC-1 was high between MM.1S and MM.1/R10R (Pearson correlation = 0.9806, p = 0.0032), and between MM.1S and MM.1R (Pearson correlation = 0.9778, p = 0.004). Similarly, the KAS-6/1 showed a similar response to B-PAC-1 as KAS-6/R10R (Pearson correlation = 0.9978, p = 0.0001) and as KAS-6/V10R (Pearson correlation = 0.9814, p = 0.003).

B-PAC-1 was cytotoxic in the presence of BCL-2 or BCL-X_L overexpression

As described above, B-PAC-1 induces apoptosis by targeting procaspase-3 and chelating inhibitory zinc ions from procaspase-3 allowing it to auto-activate. This mode of B-PAC-1 action would suggest that inactivating gene mutations or overexpression of proteins upstream in the apoptosis pathway would not affect B-PAC-1-mediated apoptosis. To test this hypothesis, we use HL-60 cell lines that overexpressed BCL-2 (HL-60/BCL-2) or BCL-X_L (HL-60/BCL-X_L) and compared the sensitivities of these cell lines to vector control HL-60 cell line (HL-60/Neo) (Fig. 6A). As shown in Fig. 6B, increasing doses of B-PAC-1 decreased HL-60/Neo, HL-60/BCL-2 and HL-60/BCL-X_L cell numbers in a dose-dependent manner. Furthermore, decreases in cell number was associated with decreases in cell viability in these 3 cell lines, as determined by measurement of annexin V+/PI+ cells (Fig. 6C). These data indicated that B-PAC-1 can bypass mutational inactivation of apoptosis that occurs due to overexpression of cell survival proteins, and induce apoptosis.

B-PAC-1 was cytotoxic to mouse embryonic fibroblasts that were wild type or deleted for MCL-1

To examine if B-PAC-1 action was independent of MCL-1, we used mouse embryonic fibroblasts that were either wild type for MCL-1 (WT MCL-1) or deleted for MCL-1 (MCL-1^{-/-}) [25]. Treatment of WT MCL-1 and MCL-1^{-/-} cells with B-PAC-1 demonstrated that it reduced cell numbers in a dose-dependent manner (Fig. 6D). The decrease in cell numbers was associated with decrease in the percentage of viable cells (cells staining negative for annexin V and propidium iodide) indicating that B-PAC-1 induced apoptosis in these cell lines (Fig. 6E).

B-PAC-1 was cytotoxic to primary myeloma cells

Finally, we examined the cytotoxicity of B-PAC-1 in primary CD138⁺ myeloma cells isolated from bone marrow aspirates of myeloma patients. CD138⁺ cells were treated with DMSO (control cells) or 20 μ M B-PAC-1 for 72 hours. Cell apoptosis was measured by staining for annexin⁺/PI⁺ cells. Higher cell death was induced by 20 μ M B-PAC-1 compared to control -treated cells (Fig. 7). These data suggest that B-PAC-1 is cytotoxic in patient myeloma cells.

Discussion

In this study, we investigated the induction of apoptosis in multiple myeloma cells using a novel small molecule procaspase-activating compound called B-PAC-1. Our data showed that B-PAC-1 induced apoptosis in MM.1S and U266 cells in a dose- and time-dependent manner. Cell death was minimal in normal B cells and peripheral blood mononuclear cells [26]. These data indicated that PAC agents such as B-PAC1 may have therapeutic efficacy in myeloma cells.

Executioner procaspases (3, 6, and 7) are cysteine-aspartic acid proteases that once activated, catalyze the terminal step in the apoptosis pathway. These proteases exist as dimers of inactive proenzymes and have to be cleaved at specific aspartic acid residues to be activated. Active caspase-3 (-6, or -7) cleaves numerous substrates that eventually lead to cell death.

Procaspace-3 is a proenzyme that is inhibited by zinc ions [6, 27]. It was previously shown that the PAC agent PAC-1 activated procaspase-3 through chelating inhibitory zinc ions [6]. This allowed procaspase-3 to auto-activate to caspase-3. Our studies show that B-PAC-1 also functions as a zinc chelator in multiple myeloma cells. 100 μ M zinc was able to inhibit B-PAC-1-mediated cell death in MM.1S cells. Furthermore, immunoblot analysis indicated that this was due to the inhibition of the formation of active caspase-3 as zinc addition to B-PAC-1-treated cells reduced the levels of active caspase-3 and increased the levels of procaspase-3.

B-PAC-1 chelates zinc ions specifically from procaspase-3 and other executioner procaspases, thereby activating apoptosis [26]. Consistent with this statement, in biochemical assay systems, B-PAC-1 did not alter the activity of other zinc-dependent enzyme such as carboxypeptidase A and HDAC [26]. Similarly, inflammatory procaspase

(procaspase-1) was not affected by B-PAC-1 [26]. These studies suggest that B-PAC-1 may not have off-target effects on other zinc-dependent enzymes.

Our studies show that B-PAC-1 reduced the levels of the IAP proteins cIAP1, cIAP2 and XIAP. We also observed decreases in the levels of the BCL-2 family survival protein MCL-1 upon treatment with B-PAC-1. These proteins are involved in myeloma cell survival [10, 13, 15–17]. Therefore, these results further indicate that B-PAC-1 reduces the survival of myeloma cells.

The bone marrow microenvironment is a major source of drug resistance in myeloma therapeutics. Soluble factors released by the bone marrow stimulate the growth and survival of myeloma cells [28, 29]. IL-6 is one such factor that is known to induce proliferation of myeloma cells and resistance to drugs [30–32]. IL-6 and similar factors induce BCL-2 family-dependent [13, 33] and –independent [34] survival pathways in myeloma cells. We showed that B-PAC-1 is equally effective in inducing apoptosis in MM.1S and U266 cells, irrespective of the presence or absence of IL-6.

HGF is another growth factor that has been implicated in myeloma disease. Myeloma patients have high levels of serum HGF, when compared with healthy donors [35], which has been correlated with poor prognosis [36–38]. Studies in our lab have further revealed that HGF levels increase as myeloma disease progresses from MGUS to SMM to newly diagnosed MM to Relapsed/Refractory MM [39]. Our studies show that B-PAC-1 effectively induced apoptosis in the presence of excess exogenous HGF.

MM is currently treated with glucocorticoids such as dexamethasone, immunomodulatory drugs such as lenalidomide, and proteasome inhibitors such as bortezomib. However, many patients who are on these treatments eventually acquire resistance to these therapies leading to disease relapse. The MM.1R cell line acquires resistance to dexamethasone because it expresses low levels, including alternative splice variants, of the glucocorticoid receptor [23, 40]. Therefore, this cell line does not undergo cytolysis upon glucocorticoid treatment. The resistance phenotype of the KAS-6/R10R lenalidomide-resistant cell line has been attributed to activation of the Wnt/ β -catenin pathway [22] and also, the HGF/MET pathway [41]. For acquired resistance to bortezomib, several factors have been implicated including upregulation of the anti-apoptotic protein MCL-1 and downregulation of the proapoptotic protein BIM [42, 43]. Our studies indicate that B-PAC-1 treatment is able to counter these resistant mechanisms and is equally effective in inducing apoptosis in myeloma cell lines that are either sensitive or resistant to these agents.

Since B-PAC-1 targets at the level of procaspase-3, we hypothesized that overexpression of survival proteins upstream in the apoptosis pathway would have no effect on B-PAC-1-mediated apoptosis. We showed that B-PAC-1 was equally effective in inducing apoptosis in cell lines irrespective of the level of BCL-2 or BCL-X_L. These data demonstrate that targeting myeloma cells at the level of procaspase-3 is an effective anti-myeloma therapy for cells that are mutated or overexpress upstream survival proteins in the apoptosis pathway. Consistent with these results, lack of BAX and BAK did not impact B-PAC-1-mediated cytotoxicity [26], further demonstrating a mechanism of cell death that is oblivious to the

BCL-2 family-defined process, an important attribute since several studies have documented resistance mechanisms in myeloma cell lines due to overexpression of BCL-2 family proteins [44–47]. Furthermore, compared to myeloma cell lines, malignant myeloma plasma cells show even higher levels of these factors [48]. Consequently, agents that neutralize BCL-2 family antiapoptotic proteins such as ABT- 737 [49] or obatoclax [48] were effective in inducing cell death in most primary patient samples. However, there was resistance to ABT-737 in samples with high MCL-1 and obatoclax was also cytotoxic to normal bone marrow cells. Bypassing these pathways using procaspase activating compounds appears to be a novel alternative approach.

We examined if B-PAC-1 can effectively induce apoptosis in primary CD138+ myeloma cells obtained from bone marrow aspirates of patients. Our data shows that myeloma cells are highly sensitive to B-PAC-1 and showed statistically significant increases in apoptotic cells compared to control cells. These studies therefore indicate that B-PAC-1 might have therapeutic efficacy in myeloma treatment.

In conclusion, we have tested a novel agent, B-PAC-1, that targets the terminal executioner procaspases, induces apoptosis in chemo-resistant and sensitive myeloma cell lines, and obviates upstream survival signal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The procaspase activating compound B-PAC-1 activates procaspase-3, -6 and -7.
2. B-PAC-1 is cytotoxic to chemotherapy-naïve and –resistant myeloma cells.
3. B-PAC-1 is cytotoxic in presence of hepatocyte growth factor and interleukin 6.
4. The action of B-PAC-1 is inhibited by exogenous zinc in myeloma cells.
5. Original PAC-1 is in phase I trial; B-PAC-1 (an analog) is evaluated for clinic.

Figure 1. Multiple myeloma cell lines express procaspase-3, -6 and -7

Immunoblot analysis of procaspase-3, -6 and -7 in multiple myeloma cell lines. GAPDH was used as internal loading control.

A

B

Figure 2. Multiple myeloma cell lines are sensitive to B-PAC-1-induced apoptosis

Multiple myeloma cell lines, MM.1S (A) and U266 (B), were treated with indicated concentration of B-PAC-1. Cells were stained for annexin V and propidium iodide (PI) and cell death was measured by flow cytometry. MM.1S (C) or U266 (D) cells were treated with indicated concentration of B-PAC-1, PAC 1 or PAC1a and cytotoxicity was measured as in A and B. Data are an average of three independent experiments and are displayed as Mean $\pm$ SEM. MM.1S (E) and U266 (F) cells were treated with 0, 3, and 10 μ M B-PAC-1 for 3, 6, and 24 hours. Cells were stained as in (A and B) and % viable cells were measured by flow

cytometry. Data are an average of three independent experiments and are displayed as Mean $\pm$ SEM.

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Figure 3. B-PAC-1-induced apoptosis by cleaving procaspase-3, -6 and -7 and its action is inhibited by exogenous zinc

(A) MM.1S cells were treated with 0, 3, and 10 μM B-PAC-1, with and without zinc, for 24 hours. Cells were also treated with 100 nM Staurosporin (a positive control for apoptosis), with and without zinc. Cells were stained for annexin V and PI and % viable cells were measured by flow cytometry. % viable cells represent the percentage of annexin V+ and PI+ cells. Data are presented as Mean ± SEM, n = 3. (B) Immunoblot analysis of MM.1S cells treated with 0, 5, and 10 μM B-PAC-1 with and without zinc for 24 hours. GAPDH was used as loading control. Caspase-3 positive and negative controls were used to identify the

cleaved caspase-3 bands. (C) MM.1S cells were treated with 0, 3 or 10 μ M B-PAC-1 for 24 hours, or treated with 100 μ M ZnSO₄, 10 μ M B-PAC-1 or 10 μ M B-PAC-1 + 100 μ M ZnSO₄ for 24 hours. Cell lysates were prepared and immunoblot analysis was done for procaspase-7 and caspase-7. Positive and negative controls were used to identify the cleaved bands. (D) MM.1S cells were treated with 0, 3 or 10 μ M B-PAC-1 for 24 hours. Immunoblot analysis was done for procaspase-6 and caspase-6. Positive and negative controls were used to identify the cleaved bands. GAPDH was used as internal loading control in all the immunoblots.

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Figure 4. B-PAC-1 induces apoptosis in the presence of IL-6 and HGF

MM.1S (A) and U266 (B) cells were treated as indicated for 24 hours. Cells were stained for annexin V and PI and % viable cells were measured by flow cytometry. Viable cells represent the percentage of annexin V⁺ and PI⁺ cells. Data are presented as Mean $\pm$ SEM, n = 3. **** $p < 0.0001$ by 1-way ANOVA when compared to DMSO-treated cells.

Figure 5. B-PAC-1 induces apoptosis in drug resistant cell lines

MM.1S (A), KAS-6/1 (B), lenalidomide-resistant MM1/R10R (C), lenalidomide-resistant KAS-6/R10R (D), dexamethasone-resistant MM.1R (E), and bortezomib-resistant KAS-6/V10R (F) cells were treated with 0, 1, 3, 10 and 20 μ M B-PAC-1 for 24 hours and % viable cells was assessed after flow cytometry analysis of annexin V+/PI+ cells. Data are Mean $\pm$ SD of 3 biological replicates.

Figure 6. B-PAC-1 induces apoptosis in HL-60 cell lines overexpressing BCL-2 or BCL-X_L and in mouse embryonic fibroblasts with wildtype or MCL-1

(A) Immunoblot analysis of HL-6/Neo, HL-60/BCL-2 and HL-60/BCL-X_L cell lines.

Immunoblots were probed for BCL-2 and BCL-X_L. GAPDH was used as loading control.

(B) HL-6/Neo, HL-60/BCL-2 and HL-60/BCL-X_L cell lines were treated with 0, 1, 3, 10, and 20 μM B-PAC-1 for 24 hours. Cell numbers were measured and are displayed as % of control. Data are represented as Mean ± SEM, n = 3. (C) HL-6/Neo, HL-60/BCL-2 and HL-60/BCL-X_L cell lines were treated as in (B). Viable cells were determined after flow cytometry analysis of annexin V+/PI+ cells. Data are displayed as % of control, Mean ±

SEM, n = 3. (D) MEF wild type (WT) for MCL-1 or lacking MCL-1 (MCL-1^{-/-}) were treated with 0, 1, 3, 10, and 20 μ M B-PAC-1 for 24 hours. Cell numbers were measured and are displayed as % of control. Data are represented as Mean $\pm$ SEM, n = 3. (E) MEF wild type (WT) for MCL-1 or lacking MCL-1 (MCL-1^{-/-}) were treated as in (D). Viable cells were determined after flow cytometry analysis of annexin V+/PI+ cells. Data are displayed as % of control, Mean $\pm$ SEM, n = 3.

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Figure 7. B-PAC-1 induces apoptosis in CD138+ primary myeloma cells

Mononuclear bone marrow cells were obtained and CD138+ cells were isolated as described in Methods. Cells were treated with 0 or 20 μM B-PAC-1 for 72 hours. Cell viability was assessed by flow cytometry analysis of annexin V+/PI+ cells. Data are displayed as ratio of control. $n=11$, $p < 0.05$

Table 1

Myeloma cell lines used in this study.

Cell Line	Origin	Medium + Supplements	Characteristics
U266	ATCC	RPMI-1640 + 10% FBS	
MM.1S	Dr. Rosen, Lurie Comprehensive Cancer Center, Chicago, IL	RPMI-1640 + 10% FBS	Glucocorticoid-and lenalidomide-sensitive
MM.1R	Dr. Rosen, Lurie Comprehensive Cancer Center, Chicago, IL	RPMI-1640 + 10% FBS	Glucocorticoid-resistant
MM.1/R10R	Dr. Orłowski, UT MD Anderson Cancer Center, Houston, TX	RPMI-1640 + 10% FBS, 1% penicillin-streptomycin, 10 mM L-glutamine, 5 µg/ml gentamycin, 10 nM lenalidomide	Lenalidomide-resistant
RPMI-8226	ATCC	RPMI-1640 + 10% FBS, 1% penicillin-streptomycin, 10 mM L-glutamine	
ANBL-6	Jelinek DF <i>et al.</i> , Cancer Research 1993;53:5320–5327	RPMI-1640 + 10% FBS, 1% penicillin-streptomycin, 10 mM L-glutamine, 1 ng/ml IL-6	Bortezomib-sensitive
ANLB-6/V10R	Dr. Orłowski, UT MD Anderson Cancer Center, Houston, TX	RPMI-1640 + 10% FBS, 1% penicillin-streptomycin, 10 mM L-glutamine, 1 ng/ml IL-6, 10 nM bortezomib	Bortezomib-resistant
KAS-6/1	Westendorf JJ <i>et al.</i> , Leukemia 1996;10:866–876.	RPMI-1640 + 10% FBS, 1% penicillin-streptomycin, 10 mM L-glutamine, 1 ng/ml IL-6	Bortezomib- and lenalidomide-sensitive
KAS-6/V10R	Dr. Orłowski, UT MD Anderson Cancer Center, Houston, TX	RPMI-1640 + 10% FBS, 1% penicillin-streptomycin, 10 mM L-glutamine, 1 ng/ml IL-6, 10 nM bortezomib	Bortezomib-resistant
KAS-6/R10R	Dr. Orłowski, UT MD Anderson Cancer Center, Houston, TX	RPMI-1640 + 10% FBS, 1% penicillin-streptomycin, 10 mM L-glutamine, 1 ng/ml IL-6, 10 nM lenalidomide	Lenalidomide-resistant