A novel vascular disrupting agent plinabulin triggers JNK-mediated apoptosis and inhibits angiogenesis in multiple myeloma cells

Short Title: Anti-myeloma activity of plinabulin

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Abstract

Previous studies have established a role of vascular disrupting agents (VDAs) as anti-cancer agents. Plinabulin is a novel VDA that exhibits potent interruption of tumor blood flow due to the disruption of tumor vascular endothelial cells, resulting in tumor necrosis. In addition, plinabulin exerts a direct action on tumor cells, resulting in apoptosis. In the present study, we examined the anti-myeloma (MM) activity of plinabulin. We show that low concentrations of plinabulin exhibit a potent anti-angiogenic action on vascular endothelial cells. Importantly, plinabulin also induces apoptotic cell death in MM cell lines and tumor cells from MM patients, associated with mitotic growth arrest. Plinabulin-induced apoptosis is mediated through activation of caspase-3, caspase-8, caspase-9, and PARP cleavage. Moreover, plinabulin triggered phosphorylation of stress response protein c-Jun amino-terminal kinase (JNK), as a primary target whereas blockade of JNK using a biochemical inhibitor or small interfering RNA (siRNA) strategy abrogated plinabulin-induced mitotic block or MM cell death. Finally, in vivo studies show that plinabulin was well-tolerated and significantly inhibited tumor growth and prolonged survival in a human MM.1S plasmacytoma murine xenograft model. Our study therefore provides the rationale for clinical evaluation of plinabulin to improve patient outcome in MM.
Introduction

Multiple myeloma (MM) is still an incurable malignancy due to the development of drug resistant phenotype after prolonged therapy. Several studies using various cancer models, including MM has provided evidence of therapeutic potential for vascular disrupting agents (VDAs). VDAs disrupt functional tumor vasculature, reducing tumor blood flow and thereby causing tumor collapse with subsequent anoxia and tumor regression. The importance of the vascular network as a therapeutic target to inhibit tumor growth has led to the development of novel VDA; that act in a ligand-directed manner and prevent tubulin polymerization (e.g. plinabulin, fosbretabulin, ABT=751), which clearly differentiates them from the microtubule-stabilizing agents (e.g., taxanes and epothilones).

Several, tubulin-stabilizing agents have been approved by FDA for the treatment of different cancer including breast, testicular and ovarian cancer, as well as Kaposi sarcoma. However, in MM there has been little clinical success with docetaxel and paclitaxel. All of these agents showed very modest anti-MM activity, associated with severe toxicity. Mechanistic studies shows that all of these VDAs target tubulin but at different site and therefore also known as tubulin poison or mitotic spindle poison. There are dozens of tubulin poison are under investigation for their anti-tumor activity but only fewer
of them are success into clinics. One possibility for their failure could be due to poor therapeutic index or imbalance between efficacy and toxicity\textsuperscript{18,19,20}

A recent preclinical study showed that small molecule tubulin polymerization inhibitor CYT997 induces MM cell death \textit{in vitro}; however, its clinical activity remains to be evaluated. Recent medicinal chemistry efforts at Nereus Pharmaceuticals, Inc led to the discovery and development of a novel VDA plinabulin (NPI-2358). Plinabulin is a synthetic analog of the diketopiperazine phenylahistin (halimide) discovered from marine and terrestrial \textit{Aspergillus} sp. Plinabulin is structurally different from colchicine and its combretastatin like analogs (e.g. fosbretabulin), and binds at or near the colchicine binding site on tubulin monomers. Previous studies showed that plinabulin induced vascular endothelial cell tubulin depolymerization and monolayer permeability at very low concentration compared to colchicine, and induced apoptosis in Jurkat leukemia cells. In addition, a phase-1 study of plinabulin as a single agent in patients with advanced malignancies (lung, prostate and colon cancer) showed a favorable pharmacokinetic, pharmacodynamic and safety profile; and a Phase-2 study combining plinabulin with docetaxel in non-small cell lung cancer patients showed encouraging safety, pharmacokinetic, and efficacy data.\textsuperscript{21,22}

In the present study, we show that the novel VDA plinabulin induces cell death in MM cells, without affecting
viability of normal peripheral blood mononuclear cells (PBMCs). The anti-proliferative activity of plinabulin is due to its ability to trigger early mitotic arrest in MM cells. Blockade of JNK abrogated plinabulin-induced mitotic arrest or MM cell death. Moreover, we show that plinabulin inhibits tumor growth in human plasmacytoma mouse xenograft models, at well-tolerated doses. These preclinical studies provide the rationale for the development of plinabulin as a novel therapy to improve patient outcome in MM.
Material and Methods

Cell Culture Human MM cell lines MM.1S, MM.1R, RPMI-8226, and INA-6 were cultured in complete medium (RPMI-1640 media supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine). MM patient tumor cells were purified by CD138 positive selection using the Auto MACS magnetic cell sorter (Miltenyi Biotec Inc., Auburn, CA). Informed consent was obtained from all patients in accordance with the Helsinki protocol. PBMCs from normal healthy donors were maintained in culture medium, as mentioned above. Plinabulin was obtained from Nereus Pharmaceuticals, Inc., San Diego, CA. Stock solution were made in 100% DMSO and stored in amber vials at -80°C.

Cell Viability and Apoptosis Assays Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International Inc., Temecula, CA), as previously described. Percent cell death in control vs. treated cells was determined by trypan blue exclusion assay. Apoptosis was assessed by Annexin V/Propidium iodide (PI) staining assay kit, as per manufacturer’s instructions (R&D Systems, Inc. Minneapolis, MN), and analyzed on a FACS-Caliber (Becton Dickinson, San Jose, CA). To examine the effects of plinabulin on mitosis, MM cells were stained with antibody against phospho-histone H3 (Cell Signaling, Beverly MA), and analyzed by flow cytometry.

Thymidine incorporation assay for co-culture experiments
cells were incubated in the presence or absence of BM stroma cells, pulsed with 3[H]-thymidine (0.5 µCi), harvested and counted by using LKB betaplate scintillation counter (Wallac)\textsuperscript{25}

**Western Blot Analysis** Protein lysate from control and drug-treated cells were subjected to immunoblotting against PARP, caspase-8, caspase-9, caspase-3, pJNK or GAPDH antibodies. (Cell Signaling, Beverly, MA) Blots were then developed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

**Small interfering RNA (siRNA) Transfection**

The JNK siRNA knockdown was performed using signal silence SAPK/JNK siRNA kit (cell Signaling, Beverly, MA). Transfection was performed using cell line Nucleofactor Kit V solution (Amaxa Biosystems/Lonza), as per the manufacturer's instructions. In brief, MM.1S cells were transfected with JNK-I or JNK-II siRNA (Cell Signaling) and then separated them into two groups. The first groups of cells were cultured for 72h; protein lysates were then analyzed for the expression of JNK-I or JNK-II by immunoblotting using anti-JNK Ab. In the second group MM.1S cells were transfected with JNK siRNA; after 24h incubation, plinabulin (8nM) was added for an additional 48h, followed by analysis of viability using MTT assay.

**Immunofluorescence assay** Cells were grown in four well chambers slides (BD Falcon, Bedford, MA) and treated with plinabulin (8nM) for 24h. Cells were then washed twice in
PBS; fixed in 0.4% of Para formaldehyde for 15min at RT; permeabilised in 0.05% triton X 100 for 5min; and blocked in blocking buffer (3% BSA+0.05% triton X 100). Fixed cells were incubated with primary Ab (alpha tubulin [1:2000]/pericentrin [1:1000]), and secondary antibody (anti-mouse Alexa 488 [1:2000]/anti-rabbit alexa 568 [1:1000]) (Invitrogen, Carlsbad, CA) for 1h each; and then washed with PBS; and mounted with prolong gold anti-fade mounting medium. (Invitrogen) Hoechst was used to stain the nuclei.

**Human Plasmacytoma Xenograft** The DFCI Institutional Animal care and Use Committee approved all animal studies. The in vivo anti-MM activity of plinabulin was assessed using the xenograft tumor model, as previously described. Briefly, CB-17 male SCID-mice (n = 12) (Charles River Labs, MA) were subcutaneously injected with MM.1S cells (5.0 x 10^6) in 100 μl of serum free RPMI-1640 medium. When tumors were measurable (~350-400 mm³) approximately two weeks after cell injection, mice were treated intraperitoneally (IP) with plinabulin (7.5 mg/kg) or vehicle alone for 21 days on a twice-weekly schedule (Day1/Day4). The dosing vehicle for plinabulin consisted of 12% PEG 400 (polyethylene glycol 400, Fluka, Sigma), 8% solutol, and 80% of double distilled water and stored in amber vials.

**In situ detection of apoptosis and assessment of microvessel density (MVD)** Tumors from untreated- and plinabulin-treated mice were excised, and analyzed for
vascular integrity and apoptosis by IHC staining using antibodies against Factor VIII and cleaved caspase-3, respectively as previously described \(^{27, 29}\).

**Statistical Analysis** Statistical significance of differences observed in drug-treated vs. control cultures was determined using the Student’s t test. Tumor volume differences were determined using a Student’s t test. The minimal level of significance was \(P < 0.05\). Survival of mice was measured using Kaplan-Meier curves (Graph Pad Software, San Diego, CA).
Results

Anti-MM activity of Plinabulin

Human MM cell lines MM.1S, MM.1R (Dex-resistant), RPMI-8226 and INA-6 (IL-6 dependent) were treated with plinabulin at different concentrations (range: 1nM-10μM) for 24h, 48h, and 72h, and cell viability was measured by MTT assays. As shown in Fig 1A, plinabulin significantly decreases the viability of all the MM cell lines in a time- and dose-dependent manner (IC50 ranges from 8-10nM for different cell lines). Importantly, plinabulin-induced decrease in cell viability was due to apoptosis, as evidenced by a significant increase in number of annexin V+/PI- cells (Fig 1B).

Plinabulin triggers mitotic arrest in MM cells

VDAs are known to block the polymerization of tubulin monomers into newly formed of microtubules and microfilaments, thereby causing mitotic arrest. Phosphorylation of histone H3 is a marker for mitotic progression and our data show that plinabulin triggers > 4 fold increase in the number of phospho-histone H3-positive cells as early as 6h post treatment. However, after 48h of plinabulin treatment the number of histone-positive cells declined with a concomitant increase in percentage of dead cells (Fig 2A). These data indicate that plinabulin induces mitotic failure early, whereas longer exposure leads to cell death. In agreement with our phospho-histone H3 data, plinabulin blocked cells in metaphase with irregular chromosome alignment (Fig 2B). Confocal images show fluorescence intensity of alpha-tubulin
(green) to be significantly down regulated in plinabulin-treated cells compared to vehicle-treated control cells (Fig 2C). These data suggest that plinabulin disrupts the formation of microtubules and microfilaments, and causing mitotic arrest in proliferating MM cells.

**Plinabulin inhibits tubule formation and migration of endothelial as well as myeloma cells.** To examine the anti-vascular activity of plinabulin, we performed capillary tubule formation assays using HUVECs. Even low concentrations of plinabulin (5nM treatment for 12h) triggered significantly decreased tubule formation in HUVECs. (Fig 3A) (70-80% decrease; P < 0.05, n=3). To further confirm the anti-vascular activity of plinabulin, we examined the effects of plinabulin on the chemo-tactic motility of both MM and endothelial cells by using trans-well insert assays. A marked reduction in serum-dependent migration in plinabulin treated MM cells (Fig 3B, 42 ± 2.1% inhibition in plinabulin-treated vs. control; P < 0.05). At this concentration 5nM for 12h plinabulin did not affect survival of MM cells (> 95% viable cells). Similar effects of plinabulin were noted against HUVEC cells (Fig 3C, 48 ± 1.7% decrease in migration; P < 0.05). Together, these data suggest that plinabulin disrupts tumor vasculature by inhibiting both cell migration and endothelial cell tubule formation.

**Plinabulin induces cell death in patient MM (CD138') cells without effecting viability of normal mononuclear
cells Patient MM (CD138') cells were treated with plinabulin (8nM, 48h), and cell viability was measured by using trypan blue exclusion assays. Two patients were newly diagnosed, and other two patients were undergoing therapy. As noted in MM cell lines, plinabulin induced significant cell death in patient MM cells (Fig 4A n=3; P<.05). Importantly, a minimal decrease in viability of normal PBMCs was observed. (Fig 4B) These results indicate potent anti-MM activity of plinabulin and suggest a favorable therapeutic index.

Plinabulin blocks BMSCs-induced MM cell growth MM.1S and MM.1R MM cells were treated with plinabulin (8nM, 48h), in the presence or absence of BMSCs; cell proliferation was then analyzed by thymidine incorporation assay. As shown in Fig 4C, plinabulin significantly inhibits BMSCs-induced MM cell growth (P < 0.05; n = 3). Our prior studies showed that adhesion of MM cells to BMSCs triggers transcription and secretion of MM cell growth and survival factors such as interleukin-6 (IL-6). We therefore next examined whether plinabulin retains its anti-MM activity even in the presence of IL-6. MM.1S and MM.1R cells were treated with plinabulin (8nM, 48h) in the presence or absence of IL-6 (10ng/ml); cell proliferation was then analyzed by thymidine incorporation assay. Results (Fig 4D) show that plinabulin induces MM cell death even in the presence of IL-6.

Plinabulin-induced apoptosis is associated with activation of caspases MM.1S, MM.1R, and RPMI-8226 cells were treated with plinabulin (8nM, 48h), and protein lysates
were subjected to immunoblot analysis. Plinabulin triggers PARP cleavage in all the three MM cells (Fig 5A). Furthermore, plinabulin induces activation of caspase-3, caspase-8 and caspase-9 (Fig 5A). These findings indicate involvement of both intrinsic (caspase-8 mediated) and extrinsic (caspase-9 mediated) apoptotic signaling during plinabulin-induced MM cell death.

**Plinabulin induced apoptosis in MM cells requires JNK**

Cell death or apoptosis induced by plinabulin can also be regulated through a stress-inducible regulatory network that includes activation of c-jun amino terminal kinase JNK. We therefore next examined effects of plinabulin on phosphorylation of JNK. Treatment of MM.1S, MM.1R and RPMI-8226 cells with plinabulin (8nM, 48h) triggered phosphorylation of JNK (Fig 5B).

To further confirm the role of JNK in plinabulin-induced cell death, we pretreated MM.1S, MM.1R, and RPMI-8226 MM cells with a biochemical inhibitor of JNK (SP600125, 20μM for 30min), followed by plinabulin treatment (10nM, 48h) and analysis of cell death using trypan blue cell viability assay. Plinabulin-induced cell death was significantly blocked (80-85%) in the presence of JNK inhibitor (Fig 5C).

We further confirmed the involvement of JNK by using siRNA strategy. Blockade of JNK-I or JNK-II using siRNA significantly abrogated plinabulin-induced apoptosis (Fig 5D and 5E). Together these data confirm an obligatory role of JNK during plinabulin-induced MM cell death.
Plinabulin induced cell death were dependent on JNK and caspase activation To further investigate the role of caspases in plinabulin induced cell death we pretreated MM.1S, MM.1R and primary patients cells with Pan caspase inhibitor (Z-VAD-FMK, 40μM, 2h) followed by plinabulin (8nM) treatment for another 48h. After desired time point trypan blue assay were performed. Our result (6A) shows that in presence of PAN caspase Inhibitor plinabulin induced cell death was significantly rescued. To further investigate if, JNK is the primary target for Plinabulin. We examined the effect of plinabulin on activation of JNK/SAPK by measuring the phosphorylation of JNK/SPNK as a function of time. To do so we treated MM cells with higher concentration of plinabulin (20nM) for different time point then we checked JNK and caspase activation by western blot. Our result (Fig 6B) shows that JNK activation occurs as early as 1h after plinabulin treatment where as caspases activated only at later time point such as 16 and 24h. All of these results together suggest JNK as a primary target for this drug. However in the presence of caspase or JNK inhibitor cells were able to rescued from cell death; so our next interest was to find out if JNK and caspases activation are dependent on each other. To investigate further about JNK or caspase dependence we treated MM cells with JNK inhibitor and checked for caspase-3 cleavage and on the other hand we treated cells with caspase inhibitor and checked for JNK activation. In both the cases caspase-3 cleavage (Fig 6C,
upper panel) and JNK activation (Fig 6C, lower panel) were blocked either in the presence of JNK or PAN caspase inhibitor which suggest that JNK and caspases are working in auto-amplification loop and their activity is dependent on each others. Our earlier result show plinabulin induced mitotic block at very early time point therefore we want to find out if JNK is also playing role in Anti-mitotic activity of Plinabulin. To do so we pretreated MM.1S cells with SP600125 and than Plinabulin was added for another 16h. Result (fig 6D) show that plinabulin induced mitotic arrest were significantly abrogated; about 50% cells were able to slippage from mitosis in the presence of JNK inhibitor which further suggest JNK as a primary target and playing major role for anti-myeloma activity of Plinabulin.

**Plinabulin inhibits tumor growth in human plasmacytoma xenograft mouse model** Having shown the in vitro anti-MM activity of plinabulin, we next examined in vivo efficacy of plinabulin by using human MM xenograft murine models. MM.1S tumor-bearing mice were treated with plinabulin (7.5mg/kg; IP) or vehicle alone twice a week for three weeks. Plinabulin treatment inhibited tumor growth and prolonged survival in mice (Fig 7A, P < 0.05). Plinabulin treatment was well tolerated, without significant weight loss or neurological changes (Fig 7B). Importantly, increased survival was noted in mice receiving plinabulin vs. vehicle alone (P =0.0041; median survival in control was 15 days vs. 35 days in the plinabulin treatment group (Fig
We next examined tumors from plinabulin-treated vs. control mice, and performed immunostaining for cleaved caspase-3, and vasculature related marker such as Factor VIII. As shown in Fig 7D, increase in cleaved caspase-3, was observed in tumor sections from plinabulin-treated group vs. controls (lower panel, Fig 7D). Moreover, anti-vascular activity of plinabulin was evidenced by a significant reduction in Factor VIII expression (Fig 7D, upper panel). These results demonstrate that in vivo anti-MM activity of plinabulin is associated with disruption of tumor vasculature and pro-apoptotic activity.
Discussion

Vascular disrupting agents (VDAs) target the cytoskeleton and tubulin network of endothelial cells, thereby causing vascular disruption and subsequent tumor cell death. Previous studies have shown that plinabulin (NPI-2358) can inhibit microtubule depolymerization in proliferating HUVECs cells at much lower concentrations compared to colchicine. Similar to colchicine, Plinabulin can inhibit polymerization of microtubule, which is an important component of tubulin and essential for mitosis therefore they also known as mitotic poison or spindle poison. Microtubule disrupting agents has ability to induce structural changes in to microtubule cytoskeleton therefore stop the cell proliferation by inducing cell cycle arrest by activating cell cycle check point that can monitors the mechanics of mitotic spindle function. Mitotic arrest is the primary function for anti-microtubule agents and responsible for cytotoxicity activity of several microtubule disrupting agents. Cells which escape form mitosis they lack both anaphase and cytokinesis and they proceed in to G1 phase of the cell cycle several studies shows that cells with intact G1 check point remains in the same state however cells with defective G1 check point undergo aberrant apoptosis or cell death.

Similar to previously reported microtubule disrupting agent Plinabulin also inhibits the proliferation of MM cells by
triggering mitotic block as an early event where as apoptosis as a terminal. \textsuperscript{11,12} Trans-well insert assays and tubule formation assays confirmed anti tumor vasculature activity of plinabulin. Plinabulin did not affect viability of normal PBMCs, suggesting a favorable therapeutic index. In MM, adhesion of tumor cells to BMSCs trigger transcription and secretion of various cytokines mediating MM cell growth, survival, migration and drug resistance \textsuperscript{30, 43, 2}; importantly, plinabulin overcomes BMSCs- or IL-6-induced MM cell growth.

Mechanistic studies showed that plinabulin-induced apoptosis is mediated through activation of caspase-3, caspase-8 and caspase-9 and PARP cleavage. Plinabulin also triggers phosphorylation of classical stress response protein JNK. Previous studies showed that sustained JNK activation is associated with apoptosis induction, whereas transient JNK activation leads to cell survival. \textsuperscript{44,45 46} Microtubule disrupting agents can induce JNK as a primary target in a variety of cancer cells by inhibiting microtubule dynamic that help cells to undergoes apoptosis. Previous studies has shown that Microtubule inhibitor can lead to proteasomal degradation of both cellular flice activating protein through mitotic arrest and JNK dependent mechanism \textsuperscript{48} and trigger sustained activation of JNK in variety of human cancer cells \textsuperscript{49} In agreement to previously reported data our result showed that JNK is getting activated very early in response to plinabulin treatment and
required for both mitotic arrest as well as apoptosis. Our results suggest that caspase and JNK both are required for Plinabulin induced cell death however JNK is appear to be a primary target. Indeed, our results showed that pharmacologic blockade of JNK-I or JNK-II with SP600125 or genetic knockdown using siRNA significantly blocked plinabulin-induced MM cell apoptosis confirming an obligatory role for JNK during plinabulin-induced mitotic arrest and MM cell death.

In vivo studies showed that plinabulin significantly inhibits tumor growth and prolongs survival of mice receiving plinabulin compared to vehicle-treated controls. Importantly, favorable tolerability was observed in mice receiving plinabulin treatment, evidenced by < 10% weight loss. Analysis of tumor sections from plinabulin treated mice showed increased apoptosis, assessed by cleaved-caspase-3 staining; associated with a marked anti-vascular activity, evidenced by reduction in Factor VIII expression. At present, plinabulin is in Phase-2 clinical trials in combination with docetaxel in NSCLC patients these in vitro and in vivo preclinical data now provide the framework for clinical trials of plinabulin to improve patient outcome in MM as well.
Acknowledgement

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Authors’ contributions
DC designed research, analyzed data, and wrote the manuscript; AVS designed research, performed experiments, analyzed data, and wrote the manuscript; MB performed the experiments; NR and PR provided clinical samples; MP designed treatment approaches, provided plinabulin and KCA wrote the manuscript.

Disclosure of Conflicts of Interest: Disclosure-DC and KC: consultant to Nereus pharmaceuticals, Inc.
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Figure Legends:

Figure 1. Plinabulin inhibits growth and triggers apoptosis in MM. (A) Human MM cell lines MM.1S, MM.1R, RPMI-8226 and INA-6 were treated with Plinabulin (dose range: .001-10μM) for 24h, 48h, and 72h; cell viability was measured using MTT assays. Data presented are mean plus or minus SD of three independent experiments (P < .05 for all the cell lines at
different time points). (B) MM.1S, MM.1R, RPMI-8226, and INA-6 cell lines were treated with plinabulin (8nM) for 48h, and apoptosis was measured using Annexin V/PI binding assay by Flow cytometry (P < 0.05; n=3,). A representative graph from three independent experiments is shown.

**Figure 2. Plinabulin treatment leads to mitotic block in MM**

(A) MM cell lines MM.1S and RPMI-8226 were treated with Plinabulin (8nM) for 6h, 24h, and 48h; followed by analysis for histone H3 phosphorylation using flow cytometry. Data is representation of three independent experiments. (B) MM.1S cells were treated with plinabulin (8nM) for 24h, and stained for α-tubulin, pericentrin and Hoechst. (C) fluorescence intensity (FI) of α-tubulin (green fluorescence) was measured by using photoshop. Representative figures (C, D) from four independent experiments.

**Figure 3. Anti-vascular activity of plinabulin**

(A) HUVECs cells were treated with plinabulin (5nM) for 12h, and assessed for in vitro vascularization using matrigel capillary-like tube structure formation assays (magnification: 4X/0.10 NA oil, media: EBM-2). Left panel: Micrograph images show the effect of plinabulin on capillary tube branch formation. Right panel: The bar graph represents quantification of capillary-like tube structure formation in response to plinabulin: Branch points in several random view fields/well were counted; values were averaged; and
statistically significant differences were measured using student’s t-test. (B, C) For migration assay, HUVECs and MM cells were treated with plinabulin (5 nM and 10nM) for 12h; cells were >90% viable at this time point. Cells were washed and cultured in serum-free medium, plated on a fibronectin-coated polycarbonate membrane in the upper chamber of transwell inserts, and exposed for 2h to serum containing medium in the lower chamber. Cells migrating to the bottom face of the membrane were fixed with 90% ethanol and stained with crystal violet (magnification: 10X /0.25 NA oil). A total of 3 randomly selected fields were examined for cells that had migrated from top to bottom chambers. Fig 3B and 3C upper panels: Bar graph represents quantification of migrated cells. Data presented are means plus or minus SD (n = 2; P < 0.05 for control versus plinabulin. Fig 3C and 3D lower panels: Image is representative of two experiments with similar results.

**Figure 4. Plinabulin induces cell death in patient tumor (CD138+) cells and inhibits BMSC-induced MM cell growth** (A) Purified patient MM cells were treated with plinabulin (8 nM) for 48h, and cell death was measured using trypan blue exclusion assays. Data presented are mean plus or minus SD of triplicate samples (P < 0.05 for all patients) (B) PBMCs from 5 normal healthy donors were treated with plinabulin (0.1 and 1μM) for 48h, and then analyzed for viability using MTT assay. Data presented are mean plus or minus SD of triplicate samples (P < 0.05; n=3). (C) MM.1S cells were
treated with plinabulin (8nM, 48h) in the presence or absence of three different patient BMSCs and cell growth was measured by using thymidine incorporation. Data presented are mean plus or minus SD of triplicate samples (P < 0.05; n=3,). (D) MM.1S and MM.1R cells were treated with plinabulin (8nM, 48h) in the presence or absence of rhIL-6 (10ng/ml), and then cell growth was measured using thymidine incorporation. Data presented are mean plus or minus SD of triplicate samples (P < 0.05, for all the cell lines).

**Figure 5 Plinabulin-induced apoptosis in MM cells is associated with activation of caspases and JNK** (A) MM.1S, MM.1R, and RPMI-8226 MM cells were treated with plinabulin (8nM) for 48h; harvested; and total protein lysates were subjected to western blot analysis using antibodies against PARP, caspase-3, caspase-8, caspase-9, or GAPDH. TL indicates total length; CF indicates cleaved fragment. Blots shown are representative of two independent experiments. (B) MM.1S and MM.1R MM cells were treated with plinabulin (8nM) for 48h; harvested; and total protein lysates were subjected to western blot analysis using antibodies against pJNK or GAPDH. Blots shown are representative of two independent experiments. (C) MM.1S, MM.1R and RPMI-8226 MM cells were pretreated with the biochemical inhibitor of JNK (SP600125, 20μM for 30min), followed by plinabulin treatment (8nM, 48h). After incubation, cell death was measured by using trypan blue assay. (N=3, P <. 05) (D) MM.1S cells were transfected with 100nM of siRNA JNK I or JNK II or scrambled
siRNA using the cell line Nucleofactor Kit V solution (Amaxa Biosystems/Lonza) for 72h, and protein expression of JNK-I or JNK-II was examined by immunoblotting with antibodies specific for JNK I and II. (E) Transfected MM.1S cells were treated with plinabulin (8nM, 48h), and cell viability was measured using MTT assay. (N=3, P>.05)

Figure 6 Cell death induced by Plinabulin is dependent on JNK as well as caspases (A) MM.1S, MM.1R and primary patients cells were pretreated with PAN caspase inhibitor Z-VAD-FMK (40μM, 2h) followed by Plinabulin treatment (8nM, 48h), After desired time point cell death were measured by using trypan blue assay. (N=2, P>.05) (B) MM.1R cells were treated with higher doses of Plinabulin (20nM) for 1h, 6h, 16h and 24h and western blot were performed by using antibodies against; pJNK, caspase-3, caspase-8, caspase-9, or GAPDH. CF indicates cleaved fragment. Results were representative of two independent experiments with similar results. (C) Upper panel: MM.1R cells were treated with PAN caspase inhibitor for 2h and then plinabulin (8nM) were added for additional 48h. Protein lysate was prepared; phosphorylation of JNK was checked, by using western blot (C) Lower panel: MM.1R cells were pretreated with JNK inhibitor SP600125 (20μM, 2h) and Plinabulin (8nM) was added for another 48h. After 48h protein lysate was prepared and cleaved caspase -3, expression was checked by using western blot. (D) MM cell lines MM.1S were pretreated with SP600125
(20μM) for 2h and Plinabulin (8nM) were added for additional 16h followed by analysis for histone H3 phosphorylation using flow cytometry. Data is representation of three independent experiments.

**Figure 7 In vivo anti-MM activity of plinabulin** (A) MM.1S cells (5 X 10^6 in 100μl of serum free RPMI-1640 medium) were implanted subcutaneously in mice (7 mice/group); average and standard deviation of tumor volume (mm^3) was monitored every third day. Mice were treated intraperitoneally (IP) with plinabulin (7.5 mg/kg) or vehicle alone twice weekly for three weeks. Bars indicate mean ± SD; P =0.05). (B) Body weight of plinabulin-treated vs. control mice were monitored once a week. Data shows plus or minus SD of 6 different mice/group. (C) Kaplan-Meier plot showing survival of mice treated with plinabulin compared to vehicle-treated controls. (D) Tumors from control and plinabulin- treated mice were subjected to immunostaining using antibodies against cleaved caspase-3 and Factor VIII. Photographs are representative of similar observations in two different mice receiving same treatment.
Figure 1

A. 24h, 48h, and 72h survival curves for Plinabulin at different concentrations in MM.1S, MM.1R, RPMI-8226, and INA-6.

B. Flow cytometry analysis of MM.1S, MM.1R, RPMI-8226, and INA-6 under control and Plinabulin treatment conditions.
Figure 2

A. Flow cytometry analysis of MM.1S and RPMI-8226 cells treated with plinabulin (8 nM) for 0h, 6h, 24h, and 48h. The graphs show the percentage of cells in different phases of the cell cycle over time.

B. Immunofluorescence images of control and plinabulin-treated cells. The images show the distribution of α-tubulin (green) and PI (red) in the cells.

C. Bar graph showing the quantification of α-tubulin fluorescence intensity (FI) in control and plinabulin-treated cells.
Figure 3

A. Diagram showing the effect of Plinabulin on the number of branch points.

B. Bar graph showing the number of cells migrated at different concentrations of Plinabulin.

C. Bar graph showing the number of migrated cells at different concentrations of Plinabulin.
Figure 4

A. Survival (%)

B. Survival (%)

C. Thymidine Uptake CPM (10^6)

D. Thymidine Uptake CPM (10^6)
Figure 5

A. Western blot analysis showing PARP, GAPDH, Caspase-3, Caspase-8, and Caspase-9 levels in MM.1S, MM.1R, and RPMI cells.

B. Western blot analysis showing pJNK levels in MM.1S, MM.1R, and RPMI cells.

C. Bar graph showing the percentage of dead cells in control, SP600125 (20 μM), Plinabulin (8 nM) treated groups.

D. Western blot analysis showing total JNK levels.

E. Bar graph showing the percentage of viable cells in control, control siRNA, JNK siRNA I, JNK siRNA II, Plinabulin, JNK siRNA I, JNK siRNA II treated groups.
Figure 6
Figure- 7

A. Tumor Volume (mm³)

B. Body Weight (gm)

C. Survival (%)

D. Images showing Factor VIII and Caspase-3 stained control and plinabulin groups.
A novel vascular disrupting agent plinabulin triggers JNK-mediated apoptosis and inhibits angiogenesis in multiple myeloma cells

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