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Halofuginone inhibits multiple myeloma growth *in vitro* and *in vivo* and enhances cytotoxicity of conventional and novel agents

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Abstract

Multiple Myeloma (MM), a malignancy of plasma cells, remains incurable despite the use of conventional and novel therapies. Halofuginone (HF), a synthetic derivative of quinazolinone alkaloid, has recently been shown to have anti-cancer activity in various preclinical settings. This study demonstrated the anti-tumour activity of HF against a panel of human MM cell lines and primary patient-derived MM cells, regardless of their sensitivity to conventional therapy or novel agents. HF showed anti-MM activity *in vivo* using a myeloma xenograft mouse model. HF suppressed proliferation of myeloma cells alone and when co-cultured with bone marrow stromal cells. Similarly, HF induced apoptosis in MM cells even in the presence of insulin-like growth factor 1 or interleukin 6. Importantly, HF, even at high doses, did not induce cytotoxicity against CD40 activated peripheral blood mononuclear cells from normal donors. HF treatment induced

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Author contribution

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accumulation of cells in the G₀/G₁ cell cycle and induction of apoptotic cell death associated with depletion of mitochondrial membrane potential; cleavage of poly (ADP-ribose) polymerase and caspases-3, 8 and 9 as well as down-regulation of anti-apoptotic proteins including Mcl-1 and X-IAP. Multiplex analysis of phosphorylation of diverse components of signalling cascades revealed that HF induced changes in P38MAPK activation; increased phosphorylation of c-jun, c-jun NH(2)-terminal kinase (JNK), p53 and Hsp-27. Importantly, HF triggered synergistic cytotoxicity in combination with lenalidomide, melphalan, dexamethasone, and doxorubicin. Taken together, these preclinical studies provide the preclinical framework for future clinical studies of HF in MM.

Keywords

Halofuginone; TGF- β ; JNK; c-jun; p-53

Introduction

Multiple myeloma (MM) is a B cell malignancy characterized by clonal proliferation of plasma cells in the bone marrow (Hideshima, *et al* 2004). Clinical features include increased risk of infection, pancytopenia, renal failure and bone disease. The use of novel anti-MM agents (such as thalidomide, lenalidomide, and bortezomib), alone or in combination with conventional chemotherapy, has improved the clinical outcome of patients; however, the development of drug resistance is universal and associated with disease progression. Thus, the pursuit for newer, next generation novel therapeutic agents remains critically important.

The small molecule halofuginone hydrobromide (HF) is a synthetic derivative of quinazoline alkaloid, originally isolated from the plant *Dichora febrifuga* (Pines and Nagler 1998). HF inhibits mainly collagen type I synthesis and extracellular matrix formation via the inhibition of transforming growth factor- β (TGF- β) signalling, and matrix metalloproteinase 2 (MMP2) (Elkin, *et al* 2000, Gavish, *et al* 2002, Liu, *et al* 1998, Nagler, *et al* 1997). Its main clinical efficacy is in scleroderma, chronic graft-versus-host disease and Kaposi sarcoma (Koon, *et al* 2011, Pines, *et al* 2003). In recent years a significant body of information has emerged supporting a critical role for interleukin 6 (IL-6) and TGF- β in MM pathobiology (Hideshima, *et al* 2007, Prabhala, *et al* 2010). In addition, several studies have shown that HF has anti-cancer activities in various tumour models (Abramovitch, *et al* 2004, Elkin, *et al* 1999a, Gavish, *et al* 2002, Genin, *et al* 2008, Gross, *et al* 2003, Nagler, *et al* 2004, Sheffer, *et al* 2007, Spector, *et al* 2010). HF has been shown to induce anti-angiogenic, anti-metastatic, and anti-proliferative effects in preclinical studies (Elkin, *et al* 2000, Taras, *et al* 2006). HF inhibits several essential stages of angiogenesis including endothelial cell proliferation, MMP2 expression, basement membrane invasion, and extracellular matrix deposition by newly formed vessels. It also inhibits collagen type-I synthesis during angiogenic sprouting (Liu, *et al* 1998). The behaviour of MM cells is determined not only by genetic features but also by their interaction with the bone marrow microenvironment (Jakubikova, *et al* 2011). Thus, targeting components of the microenvironment via the inhibition of angiogenesis, extracellular matrix and MMP2 might be an attractive option.

We have previously shown that HF inhibits the activation of nuclear factor kappa beta (NFkB), as well as tumour necrosis factor- α (TNF α) and interferon- γ secretion, in activated T cells (Leiba, *et al* 2006). A constitutive activation of NFkB pathway has been described in human myeloma cell lines and in primary myeloma cells and plays an essential role in the survival and growth of MM (Mitsiades, *et al* 2002a). Together, these data suggest that HF might have a potential activity against MM.

In the present study, we investigated the impact of HF on cytotoxicity of MM cells and phosphorylation of diverse molecules in several signalling pathways in MM cells. Our *in vitro* and *in vivo* studies showed that HF triggers growth inhibition in both MM cell lines and in patient MM cells, as well as overcomes the survival and growth advantages conferred by IL-6, insulin-like growth factor-1 (IGF-1), and bone marrow stromal cells (BMSC). Interestingly, HF enhanced cytotoxicity of conventional (melphalan, dexamethasone, and doxorubicin) and novel anti-MM (such as lenalidomide) agents. HF triggered accumulation of cells in the G₀/G₁ cell cycle, and induction of apoptosis associated with activation of several protein members of various signalling pathways including phosphorylated-c-Jun, JNK, p38 MAPK kinase, and p53. These preclinical studies provide the framework for clinical evaluation of HF, both alone and in combination, to improve patient outcome in MM.

Materials and Methods

Reagents

HF was kindly provided by Callgard Biopharmaceutical Laboratory, Sheba Medical Centre, Israel. Bortezomib and lenalidomide were obtained from Selleck (Houston, TX).

Dexamethasone, melphalan, and doxorubicin were obtained from Sigma-Aldrich (Saint-Louis, MO, USA). Recombinant human IL-6 and IGF-1 (R&D Systems, Minneapolis, MN, USA) were reconstituted with sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). TNF α was obtained from Peprotech (Rocky Hill, NJ, USA)

Primary cells and cell lines

The chemosensitive cell line RPMI 8226-S and its sublines resistant to doxorubicin (DOX; RPMI-Dox40), mitoxantrone (MIT; RPMI-MR20), and melphalan (MEL; RPMI-LR5) were kindly provided by Dr. William S. Dalton (Lee Moffitt Cancer Center, Tampa, FL). Human MM cell lines OPM-1 and OPM-2 were obtained from Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ). Dexamethasone (DEX)-sensitive MM.1S and DEX-resistant MM.1R cells were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL). All MM cell lines (RPMI-S, RPMI-Dox40, RPMI-LR5, RPMI-MR20, MM.1S, MM.1R, OPM1, OPM2, OPM6, NCI-H929, KMS11, KMS18, KMS34, JJN3, XG-1, OCIMY5, and U266) and the human stromal cell line HS-5 (American Type Culture Collection; Manassas, VA) were cultured in RPMI 1640 medium (Cellgro, Mediatech, Manassas, VA) and Dulbecco's modified Eagle medium (DMEM; Cellgro, Mediatech, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Harlan, Indianapolis, IN), 100 u/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine (GIBCO, Grand Island, NY) at 37°C in 5% CO₂, respectively.

Blood samples collected from healthy volunteers and primary MM patients were processed by Ficoll-Paque gradient to obtain blood mononuclear cells (MNCs). Primary plasma cells were purified from freshly isolated bone marrow of MM patients by positive selection using CD138 monoclonal antibody-conjugated magnetic beads according to the manufacturer's instructions (Miltenyi Biotec Inc., Auburn, CA, USA). Cells were cultured in RPMI 1640 medium containing 20% heat-inactivated FBS, 100 u/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, and maintained at 37°C in 5% CO₂. Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki.

Viability and proliferation assays

The inhibitory effect of HF, alone and combined with IL-6 or IGF-1, on the survival of MM cells was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide colourimetric cytotoxic assay (MTT; Sigma-Aldrich, St Louis, MO). Formazan crystals were dissolved by the addition of 200 µl of dimethyl sulfoxide (DMSO), and absorbance was measured at 540 and 690 nm in a Microplate reader (Dynatech Lab Inc., Chantilly, VA). The concentration of drug that inhibited cell survival to 50% (IC₅₀) was determined by Calcsyn software (Biosoft, Ferguson, MO, USA).

Viability was accessed using the CellTiterGlo (CTG; Promega, Madison, WI) assay. Purified MM patient cells were plated in 384-well plates at a density of 10,000 cells per well, and treated with increasing concentrations of HF for 48 h at 37 °C. CTG was added for 30 min, and plates were read with a Luminoskan luminometer (Labsystems, Franklin, MA).

To study the effect of HF on proliferation of MM cells cultured alone or together with BMSC, MM cells were cultured for 24 h together with BMSC in the presence or absence of HF. BMSC were seeded into the 96-well plates, 24 h prior to adding MM cells. DNA synthesis was measured by [3H]-thymidine (PerkinElmer Life and Analytical Sciences) uptake, added at 18.5GBq/well during the last 8 h of the 48-h cultures. All experiments were performed in quadruplicate.

Flow cytometry-based assays

Apoptotic cells were quantified using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis assay. Briefly, both suspension and adherent cells were collected and washed twice with cold PBS. Cells (3×10^5) were re-suspended in 100 µl of manufacturer-supplied 1X binding buffer. Then, 5 µl of Annexin V-FITC (BD Biosciences Pharmingen, San Diego, CA) and 5 µl of propidium iodide (PI) were added and incubated for 15 min in the dark at room temperature.

The mitochondrial membrane potential was studied using the JC-1 fluorescent probe to measure monomeric forms, present in cells with depolarized mitochondrial membranes, which emit only green fluorescence. Briefly, 3×10^5 cells were incubated in 200 µl of PBS/ 0.2% BSA containing 4 µM of JC-1 (Molecular Probes, Eugene, OR) for 30 min at 37 °C. After 30 min incubation in the dark at 37 °C, cells were analysed using a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA) using a 96-well format.

To determine cell cycle status of HF-treated cells vs. control cells, MM cells (3×10^5) were collected, washed twice with cold PBS and incubated in 0.05% Triton X-100 and 15 μ l RNA-se A (10 mg/ml) for 20 min at 37 °C. Then cells were cooled and incubated on ice for at least 10 min before PI (50 μ g/ml) was added. Finally, the stained cells were analysed using a FACS Canto II flow cytometer (Becton Dickinson) using a 96-well format.

Flow cytometry measurements were performed with a FACS Canto II flow cytometer equipped with 488 nm excitation laser. Fluorescence emission was measured using a bandpass filter set of 530, 585, 670 and 780 nm with respective photomultipliers FL1–FL4 required for fluorochromes. All fluorochromes were excited with a 488 nm laser, and data were collected through a respective photomultiplier, as follows: Annexin V-FITC and PI (FL1, FL3); carboxyfluorescein succinimidyl ester and PI (FL1, FL3); JC-1 (FL1, FL2, ratio FL2/FL1); cell cycle (log FL3, sub G1; lin FL3, DNA cell cycle histogram; FL3 peak versus integral for doublets discrimination); and anti-pH3 and PI (FL1, lin FL3, FL3 peak and integral). Forward/side light scatter characteristic was used to exclude the cell debris from the analysis. For each analysis, 1×10^4 cells were acquired for analysis. Data were analysed with De Novo FCS Express software (De Novo software, Los Angeles, CA, USA). Cell cycle calculations were performed with MultiCycle AV DNA analysis (Phoenix Flow Systems, San Diego, CA, USA).

Multiplex total and phosphoprotein analysis

Multiplex evaluation of total and phosphorylated levels of proteins of interest was performed with the xMAP luminex platform (Luminex Corporation, Austin, TX). A panel of 5 non-phosphorylated proteins Akt, ERK1/2, IkappaB-alpha, JNK, p38 MAPK and a panel of 16 phosphoproteins: phosphorylated Akt (Ser473), c-Jun (Ser63), ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), GSK-3alpha/beta (Ser21/Ser9), Hsp-27 (Ser78), IRS-1 (Ser636/Ser639), JNK (Thr183/Tyr185), MEK1 (Ser217/Ser221), NF-kappaB p65 (Ser536), p38 MAPK (Thr180/Tyr182), p53 (Ser15), p70 S6 kinase (Thr421/Ser424), p90RSK (Thr359/Ser363), Src (Tyr416), STAT3 (Ser727), STAT6 (Tyr641) were analysed in a 96-well format using the Bio-Plex suspension array system, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Western immunoblotting analysis

Following HF treatment, MM cells were washed twice with ice-cold PBS, resuspended in 100 μ l of ice-cold cell lysis buffer and lysed in 100 μ l of ice-cold cell lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 1 mM sodium fluoride and 1 x protease mixture), and then incubated on ice for 20 min. After centrifugation (10 min at 10,000 x g), supernatants were collected and protein concentrations were measured using a Bradford protein assay kit. Equivalent amounts of protein (20 μ g) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% non-fat dried milk in Tris-buffered saline containing 1% Tween 20 (TBS-T), followed by overnight incubation at 4 °C with 1:1000 dilution of the respective primary antibodies: poly (ADP-ribose) polymerase (PARP), caspase-3, caspase-8,

caspase-9, AIF, X-IAP, Mcl-1, Hsp-90, Hsp-70, Hsp-27, anti-phospho (p)-Hsp27 and β -actin (Cell Signaling Technology, Danvers, USA). Membranes were washed in TBS-T, and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. Proteins were visualized with the enhanced chemiluminescent (ECL) system (Amersham Bioscience, Little Chalfont, UK).

Enzyme-linked immunosorbent assay (ELISA)

Cytokine secretion and activity of caspases 3, 8 and 9 were assessed by ELISA. For coculture experiments, BMSC were seeded onto 96-well plates overnight, and MM cell lines were added to these BMSC-coated plates the following day with various concentrations of HF. After 24 and/or 48 h, supernatants were harvested and stored at -80°C . Cytokines and caspases 3, 8 and 9 were measured using the Duo set ELISA Development Kits (R&D Systems). All measurements were carried out in triplicate.

MM xenograft mice model

Severe combined immunodeficiency (SCID)-beige mice (6–8 weeks old; Charles River Laboratories Wilmington, USA) were maintained in accordance with institutional Animal Care Use Committee guidelines of Dana Farber Cancer Institute. Mice ($n=20$) were gamma-irradiated (150 rads) using Cs137 γ -irradiator source. For xenograft mice model, MM.1S cells (5×10^6 /mouse in 100 μl of PBS) were inoculated subcutaneously. When tumours reached 0.8–1 cm, mice were assigned into two treatment groups: Group 1: control (0.1 ml PBS) and Group 2: HF (0.75 mg/kg in 0.1 ml PBS) administered intraperitoneally for 5 days a week throughout the duration of experiment. Mice were monitored every 2–3 days for changes in body weight and tumour burden, measured by callipers; they were sacrificed in accordance with institutional guidelines when tumours reached 2 cm or if the mice appeared moribund, to prevent unnecessary morbidity to the mice. Mice were housed in the Animal Research Facility of the Dana-Farber Cancer Institute and experiments were performed in accordance with approved protocols.

Statistical analysis

The statistical significance of differences in drug-treated versus control cultures was determined using Student's *t* test. Data are presented as mean $\pm$ standard deviation (SD). The minimal level of significance was $P < 0.05$. Results from MTT viability assays are expressed as fraction of cells affected in drug-treated versus untreated cells. The IC_{50} of each drug was evaluated using the CalcuSyn software (Biosoft, Ferguson, MO, USA). Similarly, the interaction between HF and bortezomib, lenalidomide, melphalan, dexamethasone and doxorubicin was analysed using the CalcuSyn software program to determine whether the combination was additive or synergistic, as described previously (Chou & Talalay, 1984; Raje et al, 2004). This program is based upon the Chou-Talalay method, which calculates a combination index (CI). Analysis is performed based on the following equation: $\text{CI} = (\text{D})_1/(\text{Dx})_1 + (\text{D})_2/(\text{Dx})_2 + (\text{D})_1(\text{D})_2/(\text{Dx})_1(\text{Dx})_2$, where $(\text{D})_1$ and $(\text{D})_2$ are the doses of drug 1 and drug 2 that have *x* effect when used in combination; whereas $(\text{Dx})_1$ and $(\text{Dx})_2$ are the doses of drug 1 and drug 2 that have the same *x* effect

when used alone. Statistical analysis of *in vivo* studies was performed with Mann-Whitney Rank Sum Test, with determination of overall survival by Kaplan-Meier survival analysis.

Results

HF induces reduction of viability of MM cells regardless of sensitivity to conventional chemotherapy

We examined the effect of HF on viability of MM cell lines. MM cell lines (sensitive RPMI-S and its resistant sub-lines RPMI-Dox40, RPMI-LR5, RPMI-MR20; other MM cell lines such as MM.1S, MM.1R, OPM1, OPM2, OPM6) were treated with different concentrations of HF (6.25–400 nM) for 48 h and viability was assessed by MTT assay (Fig. 1A). HF significantly reduced MM cell line survival in a concentration-dependent manner (with IC_{50} values between 67 nM in RPMI-LR5 to 217 nM in MM.1R cell line). Similar effects of HF were observed on other MM cell lines, such as NCI-H929, KMS11, KMS18, KMS34, JIN3, XG-1, OCIMY5 cells, and U266 (Fig. 1B). Overall, HF significantly inhibited MM cell line survival in a dose-dependent manner, with an IC_{50} of around 100 nM in most MM cell lines.

To determine the effect of HF on primary MM cells, affinity-purified CD138+ cells from bone marrow samples from five MM patients were treated for 48 h with 6.25–200 nM HF (Fig. 1C). HF significantly affected viability of primary plasma cells at IC_{50} values in the range of 101–253 nM. On the other hand, a therapeutic window of HF was determined by comparison of its effects on normal peripheral blood mononuclear cells from 2 normal donors. These data confirm that HF, at the concentrations used, specifically inhibits the viability of myeloma cells while having no significant effect on normal cells.

HF induces apoptosis

Next we studied whether reduced survival of MM cells by HF was associated with apoptosis induction. To further confirm whether HF triggers apoptotic signalling in MM cells, we first measured the activity of caspases 3, 8 and 9 in MM.1S (Fig. 2A) and OPM1 (Fig. 2B) cells after 24 h incubation with HF. HF triggered caspase-3, -8 and -9 activities in these MM cells in a dose-dependent fashion, with highest activity associated with caspase-8. Apoptosis was further confirmed using flow cytometric Annexin V/PI assay showed induction of early and late apoptotic populations, followed by necrosis, in MM.1S and OPM1 cells treated with HF (50 nM) for 24 and 48 h (Fig. 2C). Next, MM cell lines (OPM2 and RPMI-S) were treated with HF (100 and 200 nM) for 24 and 48 h (Fig. 3). HF significantly increased apoptotic cells (Annexin V+/PI-) in both MM cell lines (Fig. 3A and 3B). In addition, a significant increase of necrotic cells (Annexin V+/PI+) at a dose-dependent manner was detected mainly in RPMI-S cell line (Fig. 3B).

To determine whether HF modulated mitochondrial membrane potential in MM cells, flow cytometric JC-1 dye was used (Fig. 3C and 3D). In this assay, elevated levels of JC-1 monomers directly correlated with mitochondrial membrane potential change. When exposed to HF, both OPM2 and RPMI-S cells (Fig. 3C–D) had a significant increase in JC-1 monomers in a dose-dependent manner, the latter being more sensitive to HF. Induction of apoptosis was confirmed by accumulation of the cells in sub G1 phase of the cell cycle

associated with DNA fragmentation at 24 and 48 h; again RPMI-S cells were more sensitive (Fig. 3E and 3F).

Western immunoblotting analysis (Fig. 3G) confirmed cleavage of PARP, caspase-3, -8 and decrease of caspase-9 in concentration-dependent manner in OPM2 and RPMI-S cell lines. Furthermore, HF significantly elevated the heat shock protein Hsp-90 in both MM lines tested. However, no significant changes in Hsp-70 were observed except for increased by HF in RPMI-S cells. On the other hand, Hsp-27 and p-Hsp-27 were significantly decreased by HF (p-Hsp-27 was not detected in OPM2 cells). In addition, up-regulation of the pro-apoptotic protein AIF and down-regulation of the anti-apoptotic proteins X-IAP and Mcl-1 in both MM lines was observed. In summary, these results indicated that cytotoxic activity of HF against MM cells correlated with its ability to induce apoptotic cell death in MM cells.

HF induces changes in cell cycle

The inhibitory effect of HF on MM cell lines was further characterized by changes in the cell cycle (Fig. 4). MM (OPM2 and RPMI-S) cell lines treated with HF at different concentrations showed significant accumulation of cells in G₀/G₁ cell cycle phase (Fig. 4A and 4B). This was most pronounced in RPMI-S cells at 6 h, associated with decreased S phase (Fig. 4B). Less pronounced G₀/G₁ block was detected after treatment with HF in OPM2 cells for 6–12 h. Overall, anti-MM effect of HF was associated with accumulation of cells in G₀/G₁ cell cycle arrest.

Multiplex analysis of HF-induced changes in diverse signalling pathways

Phosphorylation changes of members of diverse signalling pathways triggered by HF treatment were studied using multiplex analysis of bead array system (Fig. 5). HF treatment induced significant up-regulation of p-c-Jun in MM.1S cells. In addition, significantly increased phosphorylation of p38 MAPK kinase was noted at an early time point, followed by activation of c-jun NH₂-terminal kinase (JNK), GSK3 α/β and p53 protein at 12 h (Fig. 5A). Similar changes were detected in OPM1 cells (including activation of c-Jun, JNK, GSK3 α/β , p38 MAPK and p53) at earlier time points (6 h, Fig. 5B). Activation of Akt triggered by HF treatment was observed in OPM1 cells, while Akt dephosphorylation was observed in MM.1S cells. In addition, significant inactivation (dephosphorylation) of ERK1/2, Hsp-27, MEK1 and p70 S6 kinase were observed in HF treated in both cell lines, more pronounced in MM.1S cells. In contrast, there were no significant changes in activation of NF κ B p65, p90RSK, Src, STAT3 and STAT6 in MM.1S and OPM1 cells treated with HF.

HF overcomes paracrine MM cell growth and survival in the BM

In order to evaluate the effect of HF treatment in the BM microenvironment, MM.1S cells, cultured alone or with BMSC from MM patients, were treated with HF. HF did not confer resistance to co-culture of MM.1S cells with BMSC, as assessed by thymidine uptake (Fig. 6A). Given that IL-6 and IGF-1, which are mainly produced by BMSC, are central for MM growth and survival, we next determined whether HF maintains its cytotoxic effects against MM cells in the presence of exogenous IL-6 and IGF-1. Cytokines IL-6 and IGF-1 did not

inhibit HF-induced cytotoxic effect on MM.1S and INA6 (IL-6 dependent) cell lines, assessed by MTT assay (Fig. 6B). Our data showed that a co-culture of MM cells with BMSC, exogenous IL-6 or IGF-1, did not confer resistance to HF treatment.

***In vivo* anti-MM activity of HF**

The efficacy of HF was next evaluated in advanced xenograft model in which SCID mice were injected subcutaneously with MM.1S cells, and treatment was started when tumours reached ~1 cm. HF significantly inhibited tumour growth compared to control mice (Fig. 7A), without significant loss of weight (data not shown). Statistically significant changes were detected at day 10 of treatment ($P < 0.014$, by Mann-Whitney rank sum test) until the end of the experiment. Despite the advanced disease, Kaplan-Meier curves and log-rank analysis showed a significant increase in overall survival in the HF- treated group ($n=10$) compared with control group ($n=10$), $P=0.021$, Fig. 7B). Taken together, these data demonstrate that HF significantly inhibits MM growth and prolongs survival *in vivo*.

HF enhances cytotoxicity of conventional and novel therapies

We next examined whether HF enhances the cytotoxic effect of conventional and novel MM therapies. MM (OPM1, OPM2 and RPMI-S) cell lines were treated with combinations of HF with novel anti-MM agents (bortezomib (BRZ) and lenalidomide (LEN), as well as with conventional drugs (dexamethasone (DEX), doxorubicin (DOX) and melphalan (MEL)). The cytotoxicity of combined treatment was analyzed by MTT assays, and synergism was evaluated using CalcuSyn software (Jakubikova *et al* 2011). The fractions affected (Fa) and the combination indices (CI) for each of the combinations are summarized in Table 1. Using CalcuSyn software, we showed that HF generated moderate antagonism when combined with BRZ. On the other hand, immunomodulatory drug LEN, was synergistic in all cell lines except of RPMI-S, in which additive effects were detected (Table 1A). Furthermore, HF in combination with established conventional anti-MM agents DEX and DOX, triggered significant synergistic cytotoxicity in OPM1 and OPM2 cells as well as additive effects in RPMI-S cells (Table 1B). Importantly, the strongest synergistic activity was observed when HF was combined with MEL at 72 h (Table 1C). These data demonstrate that some combination therapies may synergistically enhance HF induced cytotoxicity in MM cells.

Discussion

HF, an orally available synthetic derivative of quinazolinone alkaloid, has shown preclinical anti-tumour activity in various models, including human hepatocellular carcinoma, sarcoma, brain, bladder, breast, and prostate cancer (Abramovitch, *et al* 2004, Elkin, *et al* 1999a, Gavish, *et al* 2002, Genin, *et al* 2008, Gross, *et al* 2003, Nagler, *et al* 2004, Sheffer, *et al* 2007, Spector, *et al*. 2010). The present study showed that HF induced MM cytotoxicity *in vitro* and *in vivo*. HF was active against MM cell lines that were sensitive and resistant to conventional and novel therapies. Importantly, HF has very similar potent cytotoxic activity against primary MM cells and in MM cell lines, without significant toxicity towards normal cells (peripheral blood mononuclear cells from normal donors), suggesting that it may preferentially kill MM cells while sparing normal cells. The IC_{50} of HF against primary MM was in the range of 101–253nM. Moreover, HF induced significant anti-tumour activity *in*

vivo, associated with significantly decreased tumour burden and statistically significant prolongation of overall survival.

How HF modulates intracellular signalling molecules leading to apoptosis is not completely understood. Several phosphorylation changes of signalling pathways have been triggered by HF treatment. HF treatment induces phosphorylation of c-Jun, as well as its upstream molecule JNK, in MM cells. Recently, induction of c-Jun phosphorylation in fibroblasts was described as an important mechanism by which HF exerts its inhibitory effect on collagen type I synthesis (McGaha, *et al* 2002). A direct association between HF-dependent inhibitions of collagen type I gene expression and tumour growth was observed in bladder carcinoma, glioma, and prostate cancer (Abramovitch, *et al* 1999, Elkin, *et al* 1999b, Gavish, *et al* 2002, Gross, *et al* 2003). In addition HF, a known inhibitor of TGF- β signal transduction also inhibits Smad-3 phosphorylation (Roffe, *et al* 2010). Previous data showed that c-Jun suppresses Smad-3-driven collagen gene expression and Smad-3 DNA binding activity (Wendling, *et al* 2003). Thus, c-Jun appears to be a strong regulator of type I collagen gene expression and Smad-3 transcriptional activity. This could be a major pathway by which HF exerts its antitumour activity.

Similar to previous studies (Tacheau, *et al* 2007), the present study found that HF induced p38MAPK and Jun N-terminal kinases (JNKs) phosphorylation (Fig.5), both of which belong to the superfamily of MAP kinases involved in regulation of cell proliferation, differentiation, and apoptosis (Dhanasekaran and Reddy 2008). As reported, JNK activation is one of the crucial pathways modulating apoptosis induced by leading anti-myeloma agents, such as proteasome inhibitors or IMiDs, as well as the novel oral alkylphospholipid perifosine (Anderson 2005, Hideshima, *et al* 2006, Hideshima, *et al* 2003a). JNKs activate apoptotic signalling either through the up-regulation of pro-apoptotic genes via transactivation of specific transcription factors such as c-Jun or by directly modulating the activities of mitochondrial pro-apoptotic proteins via phosphorylation events (Chauhan, *et al* 2003, Sluss, *et al* 1994). Signalling pathways that initiate apoptosis have been broadly classified into (1) extrinsic pathways initiated by death receptors, such as those of TNF α , TRAIL, and FAS-L; and (2) intrinsic pathways initiated by mitochondrial events (Elmore 2007). The central role of JNKs in both of these pathways, through the activation of caspases 3, 8 and 9-, is consistent with our observation that HF induced apoptosis via both pathways. Our observation that HF induced disruption of mitochondrial membrane potential as well as concentration-dependent activation of caspase-9, further supports the suggestion that mitochondria are also involved in HF-induced cell death.

An alternative pathway contributing to JNK-mediated apoptosis involves the phosphorylation of the p53 family of proteins by JNKs (Fuchs, *et al* 1998). We also found that HF significantly increased phosphorylation of p53, while down-regulating its downstream anti-apoptotic protein Mcl-1, in MM cell lines. Recently, the small molecule MDM2 inhibitors, nutlin and RITA, which activate p53, have been shown to induce apoptosis in MM cell lines and in primary MM cells. Reactivation of p53 and induction of tumour cell apoptosis was mediated through modulation of multiple apoptotic regulatory proteins, including down-regulation of Mcl-1 (Saha, *et al* 2010a). As is well known, p53 tumour suppressor is a multifunctional transcription factor that regulates cellular processes

affecting proliferation and apoptosis, and its inactivation by mutations and/or deletions occurs in nearly half of human tumours (Olivier, *et al* 2002). Importantly, accumulated experimental data suggests that p53 reactivation might be a widely applicable and efficient anti-myeloma strategy (Ooi, *et al* 2009, Saha, *et al* 2010b, Saha, *et al* 2010c, Stuhmer, *et al* 2005)

Mechanistically, we confirmed herein activation of MAPK pathways in MM triggered by HF, including downstream target JNKs and p38 MAPK. Various new candidate anti-myeloma agents, such as the histone deacetylase inhibitors, induce apoptosis by activating the p38 MAPK pathway in myeloma cells, suggesting the opportunity for a combination approach (Feng, *et al* 2007, Hideshima, *et al* 2003b, Kobayashi, *et al* 2010).

In our study, similar to a previous study in hepatic cancer cells (Gnainsky, *et al* 2006), the anti-myeloma effect of HF triggers cell cycle alteration characterized by accumulation of cells in the G₀/G₁ phase of cell cycle and induction of apoptosis. HF-induced apoptosis in MM cells was detected by annexin V staining and DNA fragmentation, and further confirmed by proteolytic cleavage of PARP, up-regulation of the pro-apoptotic protein AIF, and Hsp27, and p-Hsp27, as well as down-regulation of the anti-apoptotic proteins Mcl-1 and X-IAP.

Stromal cells in the bone marrow microenvironment interact with malignant plasma cells and stimulate proliferation and survival of MM via cytokine production and/or cell-cell contact, which in turn stimulates proliferation and survival of MM cells and is associated with attenuating the response of MM to conventional therapies (Mitsiades, *et al* 2007). However, we found that HF retains its anti-tumour activity through inhibition of MM cell (both IL6-dependent and independent) proliferation in the context of BMSC. We further observed that the cytotoxic effect of HF on MM cells growth was not attenuated by the addition of IL-6 and IGF-1. Therefore, these studies suggest that the bone marrow milieu, including bone marrow-derived cytokines and stromal cells, does not confer resistance to HF treatment.

Previous studies have shown that the interaction of MM cells with BMSC and the associated secretion of cytokines activated MEK/ERK signalling, which mediates MM-cell proliferation, survival, drug resistance, and angiogenesis (Tai, *et al* 2007). Therefore, blocking this pathway is also a promising treatment strategy. In the present study we observed significant inactivation (through dephosphorylation) of ERK1/2, MEK1 as well as inactivation of the anti-apoptotic Hsp-27, p-Hsp-27 and p70 S6 kinase. p-Hsp27 is a key element that promotes cell survival by inhibiting apoptosis. Surprisingly, although overexpression of Hsp-27 is also associated with bortezomib resistance (Mitsiades, *et al* 2002b), no synergism with bortezomib was found.

Another postulated mechanism by which HF may exert its anti-myeloma effect is via inhibition of T helper 17 (TH17) cell differentiations and IL-17 production (Sundrud, *et al* 2009). TH17 cells, characterized by their production of IL-17, have recently emerged as important mediators of proliferation, survival, and immune suppression in MM (Prabhala, *et al* 2010). Sundrud *et al* (2009) have shown that HF selectively inhibits the development of

human TH17 cells, without altering TH1, TH2, or iTreg differentiation. It will be intriguing to evaluate the effect of HF on these and other cytokines and chemokines in our models of MM cell growth, alone and in the context of the bone marrow milieu.

In recent years, drug combination therapies in MM have proven to be more efficacious than single agent therapy. Moreover, a previous study showed that HF exerts a synergistic effect when combined with conventional chemotherapy for solid tumours, including prostate cancer and Wilm's tumour (Sheffer, *et al* 2007). We therefore examined for synergy of HF therapy with established and novel anti-MM agents. Our study revealed that HF exhibited synergistic cytotoxicity in combination with conventional and novel anti-myeloma therapies including lenalidomide, melphalan, doxorubicin and dexamethasone, whereas bortezomib showed a possible slight antagonistic effect at the concentration studied when combined with HF, although this result needs to be interpreted with caution. Both lenalidomide and HF have anti-angiogenic properties, but the molecular mechanism of their *in vitro* synergy against MM cells remains to be elucidated, although the effects seen are intriguing given the propensity of both agents to modulate both intracellular signalling as well as adhesion. Overall, our studies thus provide the preclinical framework for clinical trials of combination HF therapies in MM.

In summary, our data show that HF-induced MM apoptotic cell death is mediated, at least in part, by the activation of c-Jun, JNKs, p38MAPK kinase, and p53 proteins, as well as inactivation of ERK1/2 and MEK1. Multiple survival and anti-apoptotic pathways are up-regulated in cancer cells; therefore, novel treatments that target multiple pathways rather than a single pathway might be a promising approach.

In a Phase I pharmacokinetic study, oral HF was administered to 24 patients with advanced cancer and was found to be safe and tolerable (de Jonge, *et al* 2006). The maximum tolerated dose reached 3.5 mg/day with nausea, vomiting, and fatigue as dose-limiting toxicities. Due to its favorable toxicity profile, it seems likely that HF can be readily combined with conventional and novel therapies. Taken together, our data provide the preclinical rationale for clinical trials of HF, alone and in combination, to improve patient outcome in MM.

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Figure 1. HF induces cytotoxicity in MM cells, regardless of sensitivity to conventional chemotherapy

(A) MM cell lines (RPMI-S, RPMI-Dox40, RPMI-LR5, RPMI-MR20, MM.1S, MM.1R, OPM1, OPM2, OPM6) were treated with 6.25–400 nM HF for 48 h. (B) MM cells (NCI-H929, KMS11, KMS18, KMS34, JJN3, XG-1, OCIMY5 cells, and U266) were treated with various concentrations of HF for 48 h in 96-well plates and cytotoxicity was assessed by MTT assay. (C) Freshly isolated bone marrow CD138⁺ tumour cells from 5 patients with MM and PBMCs isolated from 2 healthy donors were cultured with HF (25–200 nM) for 48 h and cytotoxicity was assessed by MTT assay. The IC₅₀ values (the concentration of the HF that reduced cell survival by 50%) were determined by the CalcuSyn software. Each treatment with a specific concentration of the compound was done in triplicate. The data presented are the mean SD of the results obtained from three replicate experiments, and expressed as relative viability of untreated controls.

Figure 2. HF induces apoptosis via activation of caspases and Annexin V induction

(A) MM1S and (B) OPM1 cells were incubated for 24 h with various concentrations of HF (25–200 nM) and measured for caspase-3, caspase-8 and caspase-9 activity. Data represent mean ($\pm$ SD) of triplicate cultures.

(C) MM.1S and OPM1 cells treated with 50 nM concentration of HF at 24 and 48 h and analysed for induction of apoptosis by Annexin V-FITC/PI assay. For each dot blot, the bottom left quadrant represents the percentage of viable cells (Annexin V-FITC-/PI-; green), bottom right quadrant represents the percentage of cells in early apoptosis (Annexin V-FITC+/PI-, blue), middle right quadrant represents the percentage of cells in late apoptosis (Annexin V-FITC+/PI+/-, purple) and the upper right quadrant of each dot blot represents the percentage of cells in necrosis (Annexin V-FITC+/PI+, red). Numbers in the quadrants of each dot blot represent the percentage of cells.

Figure 4. HF induces G₀/G₁ cell cycle arrest

(A) OPM2 and (B) RPMI-S cells were cultured with HF (50, 100 and 200 nM) for 6 and 12 h. The distribution of cells in G₀/G₁, S and G₂/M phase was analysed by flow cytometry and MultiCycle AV DNA analysis. Two independent experiments were performed, and means $\pm$ standard error are presented.

Figure 5. HF induces changes in phosphorylation of members from different signalling pathways in MM cells

(A) MM.1S and (B) OPM1 cells were exposed to either DMSO (control) or HF (50, 100 and 200 nM) for 2, 6 and 12 h. A multiplex panel of 16 phosphoproteins including Akt, c-Jun, ERK1/2, GSK-3α/β, Hsp-27, IRS-1, JNK, MEK1, NF-κappaB p65, p38 MAPK, p53, p70 S6 kinase, p90RSK, Src, STAT3, STAT6 were normalized to a panel of 5 total proteins and were analysed in a 96-well format using the Bio-Plex suspension array system. Data are from two independent experiments; fold changes compared with the control are depicted in a colour format, according to the scale shown.

Figure 6. HF overcomes the protective effect of IL-6, IGF-1 or BMSC on MM cell growth
 (A) MM.1S cells were incubated for 24 h with HF (50 and 100 nM) in the presence or absence of BMSC from MM patients. Proliferation was measured by (3H) thymidine uptake.
 (B) Serum starved MM1S and INA-6 cells were pre-incubated with HF (50, 100 and 200 nM) for 1h and then stimulated with IL-6 (10 ng/ml) and IGF-1 (50 ng/ml) and cytotoxicity was measured by MTT assay. Data were performed in quadruplicate and are representative of three independent experiments.

Figure 7. *In vivo* effects of HF in xenograft MM model

MM.1S cells (5×10^6 /mouse in 100 μ l of PBS) were injected subcutaneously in SCID-beige mice. Mice ($n=10$ per group) received HF (0.75 mg/kg in 0.1 ml PBS) or control vehicle (0.1 ml PBS), intraperitoneally once a day for 5 days. (A) Tumour diameters were measured every 2–3 days with callipers, and tumour volumes were calculated according to the formula of the volume of an ellipse: $1/2(\text{Length} \times \text{Width}^2)$. HF significantly inhibited tumour growth

($P = < 0.014$; Mann-Whitney rank sum test). (B) Kaplan-Meier analysis of overall survival confirmed that HF increased survival ($P = < 0.01$) compared with control mice.

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Table 1

HF enhances cytotoxicity of novel and conventional agents. MM (OPM1, OPM2 and RPMI-S) cells were cultured with HF (25, 50 and 100 nM) in combination with (A) 5 nM bortezomib (BRZ) or 25 μ M lenalidomide (LEN) for 48 h (B) 500 nM dexamethasone (DEX), or 250 or 500 nM doxorubicin (DOX) for 48 h (C) 10 μ M melphalan (MEL) for 24 and 72 h. Cytotoxicity was measured by MTT assay. Fractions-affected (Fa – ratio of number of nonviable MM cells/total number of MM cells) and combination indices (CI) of HF with BRZ, LEN, DEX, DOX and MEL were calculated according to the Chou-Talalay method. Data represent mean ($\pm$ SD) of triplicate cultures

A							
Combination of HF with novel anti-MM agents: bortezomib (BRZ) and lenalidomide (LEN) at 48 h							
	HF [nM]	BRZ [nM]	Fa	CI	HF [nM]	LEN [μ M]	Fa CI
OPM1	25	5	0.18	1.40	25	25	0.23 0.76
	50	5	0.28	1.42	50	25	0.38 0.75
	100	5	0.45	1.55	100	25	0.58 1.00
OPM2	25	5	0.40	1.18	25	25	0.16 0.56
	50	5	0.44	1.40	50	25	0.21 0.79
	100	5	0.60	1.35	100	25	0.51 0.79
RPMI-S	25	5	0.72	1.06	25	25	0.01 1.86
	50	5	0.67	1.41	50	25	0.13 0.96
	100	5	0.66	1.81	100	25	0.45 1.02
B							
Combination of HF with conventional anti-MM agents: dexamethasone (DEX) and doxorubicin (DOX) at 48h							
	HF [nM]	DEX [nM]	Fa	CI	HF [nM]	DOX [nM]	Fa CI
OPM1	25	500	0.21	0.68	25	500	0.56 0.51
	50	500	0.39	0.62	50	500	0.62 0.53
	100	500	0.62	0.90	100	500	0.68 0.71
OPM2	25	500	0.22	0.62	25	250	0.34 0.78
	50	500	0.40	0.70	50	250	0.35 1.11
	100	500	0.64	0.81	100	250	0.60 0.87

B									
Combination of HF with conventional anti-MM agents: dexamethasone (DEX) and doxorubicin (DOX) at 48h									
RPMI-S	HF [nM]	DEX [nM]	Fa	CI	HF [nM]	DOX [nM]	Fa	CI	
	25	500	0.08	0.86	25	250	0.34	1.08	
	50	500	0.26	0.81	50	250	0.43	1.07	
	100	500	0.56	1.03	100	250	0.64	1.09	
C									
Combination of HF with conventional anti-MM agent: melphalan (MEL) at 24h and 72h									
OPM1	HF [nM]	24h MEL [uM]	Fa	CI	HF [nM]	72h MEL [uM]	Fa	CI	
	25	10	0.14	0.76	25	10	0.46	0.57	
	50	10	0.25	0.77	50	10	0.50	0.59	
	100	10	0.34	1.12	100	10	0.61	0.62	
OPM2	HF [nM]	24h MEL [uM]	Fa	CI	HF [nM]	72h MEL [uM]	Fa	CI	
	25	10	0.11	0.66	25	10	0.60	0.58	
	50	10	0.21	0.74	50	10	0.61	0.73	
	100	10	0.39	0.89	100	10	0.72	0.69	
RPMI-S	HF [nM]	24h MEL [uM]	Fa	CI	HF [nM]	72h MEL [uM]	Fa	CI	
	25	10	0.13	0.95	25	10	0.57	0.77	
	50	10	0.20	1.03	50	10	0.59	0.76	
	100	10	0.41	0.85	100	10	0.69	0.59	