

Aminopeptidase inhibition as a targeted treatment strategy in myeloma

Hannah E. Moore,¹ Emma L. Davenport,¹ Emma M. Smith,¹ Srikanth Muralikrishnan,¹ Alan S. Dunlop,¹ Brian A. Walker,¹ David Krige,² Alan H. Drummond,² Leon Hooftman,² Gareth J. Morgan,¹ and Faith E. Davies¹

¹Section of Haemato-Oncology, Institute of Cancer Research, Sutton, Surrey, United Kingdom and ²Chroma Therapeutics Ltd., Abingdon, Oxon, United Kingdom

Abstract

Myeloma cells are highly dependent on the unfolded protein response to assemble folded immunoglobulins correctly. Therefore, targeting protein handling within a myeloma cell by inhibiting the aminopeptidase enzyme system, which catalyses the hydrolysis of amino acids from the proteins NH₂ terminus, represents a therapeutic approach. CHR-2797, a novel aminopeptidase inhibitor, is able to inhibit proliferation and induce growth arrest and apoptosis in myeloma cells, including cells resistant to conventional chemotherapeutics. It causes minimal inhibition of bone marrow stromal cell (BMSC) proliferation but is able to overcome the microenvironmental protective effects, inhibiting the proliferation of myeloma cells bound to BMSCs and the increase in vascular endothelial growth factor levels seen when myeloma cells and BMSCs are bound together. Additive and synergistic effects are seen with bortezomib, melphalan, and dexamethasone. Apoptosis occurs via both caspase-dependent and non-caspase-dependent pathways with an increase in Noxa, cleavage of Mcl-1, and activation of the unfolded protein response. Autophagy is also seen. CHR-2797 causes an up-regulation of genes involved in the proteasome/

ubiquitin pathway, as well as aminopeptidases, and amino acid deprivation response genes. In conclusion, inhibiting protein turnover using the aminopeptidase inhibitor CHR-2797 results in myeloma cell apoptosis and represents a novel therapeutic approach that warrants further investigation in the clinical setting. [Mol Cancer Ther 2009;8(4):762–70]

Introduction

Novel treatment strategies for multiple myeloma targeting protein turnover using the proteasome inhibitor bortezomib have recently been proven to be effective in the clinic, inducing complete responses and improving survival (1). The reversible inhibition of proteasome function results in myeloma cell death via several mechanisms including inhibition of nuclear factor- κ B activation (2), activation of the intrinsic and extrinsic cell death pathways (3), alteration in p53-mediated apoptosis and DNA repair mechanisms (4), alteration in the calcium balance in the endoplasmic reticulum (5), and activation of the unfolded protein response (UPR; refs. 6, 7). These observations clearly show that interfering with intracellular protein handling is an effective approach for the treatment of myeloma and suggest that targeting this pathway with other small molecules may also be effective.

One method to target protein handling within a cell is to inhibit the aminopeptidase enzyme system that catalyses the hydrolysis of amino acids from the NH₂ terminus of protein and peptide substrates. These enzymes are essential to many physiologically important processes such as protein maturation (8), degradation of peptides (9), and cell cycle control (10). Inhibiting these enzymes disrupts protein turnover leading to an accumulation of peptides and a reduction in the cellular free amino acid content, which has profound effect on cell survival and proliferation. Importantly, many tumor cells are dependent on specific amino acids and previous studies have shown that depletion of these amino acids has a greater effect on cancer cells than normal cells (11, 12). Evidence for the clinical value of such an approach is seen from the use of bestatin, a broad-range inhibitor of aminopeptidases, which has been used for over a decade in Japan for the treatment of acute and chronic acute myeloid leukemia (13, 14).

CHR-2797 (Chroma Therapeutics) is a novel inhibitor that is targeted to the M1 family of aminopeptidases, a group of metalloenzymes that contain a central Zn²⁺ ion. Members of the M1 family include the membrane-bound ectopeptidases aminopeptidase N and A together with the cytosolic enzymes aminopeptidase B, leukotriene A₄ hydrolase, and puromycin-sensitive aminopeptidase, the latter being implicated in proteasome interactions (10). CHR-2797 is a water-soluble compound that, once

Received 8/4/08; revised 12/15/08; accepted 1/11/09.

Grant support: Kay Kendall Leukaemia Fund, General Clinical Research Fund of The Royal Marsden Hospital, and Department of Health.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: H.E. Moore performed the research, analyzed the data, and wrote the article. E.L. Davenport, E.M. Smith, S. Muralikrishnan, A.S. Dunlop, and B.A. Walker performed the research and analyzed the data. D. Krige, A.H. Drummond, L. Hooftman, and G.J. Morgan designed the research and wrote the article. F.E. Davies designed and performed the research, analyzed the data, and wrote the article.

Requests for reprints: Faith E. Davies, Section of Haemato-Oncology, Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, United Kingdom. Phone: 44-208-722-4130; Fax: 44-208-722-4432. E-mail: faith.davies@icr.ac.uk

Copyright © 2009 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-08-0735

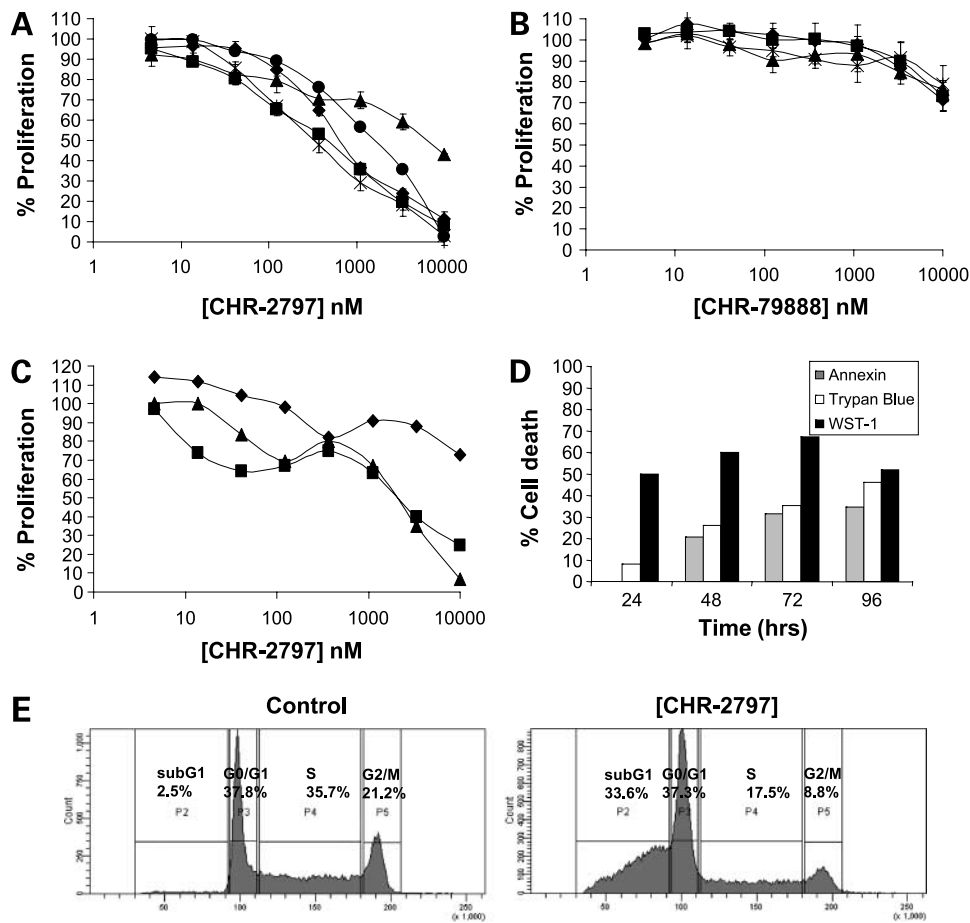


Figure 1. CHR-2797 inhibits proliferation in multiple myeloma cell lines. **A**, treatment of myeloma cell lines over a 96 h period with CHR-2797 inhibited proliferation as determined by a WST-1 assay (◆, H929; ■, MM1s; ▲, U266; ●, JJN3, and *, MM1r). Average \pm SE of three experiments. **B**, acid metabolite CHR-79888 failed to inhibit proliferation in the same assay. Average \pm SE of three experiments. **C**, CHR-2797 was able to induce cell death in CD138⁺ plasma cells isolated from patient samples over a 72 h time course (◆, patient 1; ■, patient 2; and ▲, patient 3). **D**, cell death in myeloma cell lines was validated over a 96 h time course using Annexin V/PI staining and trypan blue exclusion. Representative data of one experiment on H929 cells. All experiments were done in triplicate. **E**, CHR-2797 induced an increase in the number of apoptotic cells (in the sub-G₁ fraction) as determined by PI binding to DNA. Representative data from H929 cells following 24 h in treatment with and without CHR-2797 (650 nmol/L). The experiment was done in triplicate.

inside the cell, is susceptible to intracellular esterases that generate the charged acid metabolite CHR-79888. This also has inhibitory effects against aminopeptidases and its low membrane permeability results in intracellular accumulation. In preclinical studies, both CHR-2797 and CHR-79888 are more potent against targets such as puromycin-sensitive aminopeptidase and leukotriene A₄ than bestatin (Chroma Therapeutics; data on file; ref. 15).

As myeloma cells are reliant on protein production and the UPR (7), we hypothesized that disrupting protein turnover using an aminopeptidase inhibitor would be particularly toxic to these cells. We therefore tested the effects of CHR-2797 on a panel of myeloma cells for its ability to inhibit proliferation and induce apoptosis. We also looked at the interactions between the myeloma cells and bone marrow stromal cells (BMSC) to establish whether the cell-cell interactions are affected by drug treatment and whether CHR-2797 is able to overcome the protective effects of the

bone marrow microenvironment. Expression arrays and Western blot analysis were used to identify genes and proteins that are deregulated in the presence of CHR-2797 to further understand its mode of action and gain insight into the design of rational drug combinations.

Materials and Methods

Cell Lines and Reagents

The multiple myeloma cell lines U266, H929, MM1s, MM1r, and JJN3 were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS and GlutaMAX-I (Invitrogen), 60 μ g/mL penicillin, and 100 μ g/mL streptomycin. Cultures were maintained in exponential growth at 37°C in a humidified atmosphere of 95% air/5% CO₂. Following informed consent, bone marrow aspirates were obtained from myeloma patients and CD138⁺ plasma cells were selected using antibodies conjugated to magnetic beads on

an Automacs machine (Miltenyi Biotec). BMSCs were also obtained from patient bone marrow aspirates: cells were cultured in RPMI 1640 supplemented with 20% heat-inactivated FCS for 24 h before any nonadherent cells were removed. Adherent cells were grown for up to 4 weeks with the medium changed every 3 to 4 days. The amino peptidase inhibitors CHR-2797 and CHR-79888 (Chroma Therapeutics) were dissolved in DMSO to a stock concentration of 10 mmol/L. Bortezomib (Velcade; Johnson & Johnson), a proteasome inhibitor, was dissolved in DMSO to a stock concentration 1 mmol/L, and dexamethasone (Sigma-Aldrich) was dissolved in methanol to a stock concentration of 50 mmol/L. The caspase inhibitor Z-VAD-FMK (Calbiochem) was supplied as a 10 mmol/L solution. All experiments were done in triplicate.

Cell Proliferation, Survival, and Cell Cycle Assays

Inhibition of proliferation was measured using an *in vitro* WST-1-based assay according to the manufacturer's instructions (Roche). Following serum starvation, cells were seeded in 96-well microculture plates at a density of 5,000 per well 24 h before the addition of drug treatment. Plates were incubated in a humidified incubator in 5% CO₂ for 24 to 96 h at 37°C. Subsequently, 10 μ L WST-1 solution (final concentration 5 mg/mL) was added per well and incubated for 4 h at 37°C. Plates were read on a Dynatech Laboratories MRX plate reader.

Cell death was verified in CHR-2797-treated cells using trypan blue exclusion to determine the number of viable cells and by flow cytometry to measure Annexin V/PI (Bender Medsystems) expression on a FACSCalibur (Becton Dickinson). Where the caspase inhibitor Z-VAD-FMK was used, it was added 1 h before CHR-2797 at a concentration of 50 μ mol/L. Cell cycle status was measured in untreated and CHR-2797-treated cells after 24 h incubation. Cells were fixed in 70% ethanol, stained with propidium iodide (PI), and analyzed by flow cytometry using CellQuest Pro for cell cycle analysis. The formation of cytoplasmic inclusions was identified using May-Grunwald-Giemsa stain and visualized using an Olympus BH-2 light microscope with \times 100 oil objective magnification. Images were acquired using a JVC KY-F1030 digital camera and JVC Scan Rate Converter

software. Immunoglobulin light chain was detected using polyclonal κ and γ antibodies (DAKO) with a labeled streptavidin biotin secondary antibody (Ventana Medical Systems). Samples were processed using the Ventana benchmark XT immunostaining machine and the iView DAB detection kit.

Cytokine Measurement

BMSC and MM1s cells were grown separately using Transwell inserts (6.5 mm, pore size 0.3 μ mol/L) and together in coculture in 24 well plates at 5×10^4 cells/mL in the presence or absence of 500 nmol/L CHR-2797. After 24 h, medium was removed and analyzed for the cytokines interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) by the ELISA method using Quantikine Immunoassay kits according to the manufacturer's instructions (R&D Systems).

RNA Extraction, Amplification, and Gene Expression Measurement

RNA was extracted from treated myeloma cells using commercially available kits (Abgene) according to the manufacturer's instructions. RNA quality and quantity were determined using a 2100 Bioanalyzer (Agilent). Total RNA (100 ng) was amplified using a two-cycle target labeling kit (Affymetrix) and cRNA was hybridized to Human Genome U133 Plus 2.0 expression arrays as described previously (16). The arrays were washed on an Affymetrix Fluidics Station 450 and scanned with a Gene Chip Scanner 3000. Following normalization using Affymetrix software, data were analyzed using dChip software, which is freely available on the Web. Supervised analysis was done using dChip to determine genes differentially expressed in the control (C) or treated (T) cells; each cell line was analyzed independently. Comparison criteria used were lower bound fold change T/C >1.2 or C/T >1.2 and mean difference T-C >100 or C-T >100.

SYBR Green real-time PCR (RT-PCR; Applied Biosystems) was done on cDNA synthesized from equal quantities of RNA using the SuperScript III first-strand synthesis kit (Invitrogen). Primer sequences were designed using Primer Express 2.0 (Applied Biosystems) over exon/exon boundaries for *CHOP* F5'-TGGAATGAAGAGGAAGAATCAAAA3'

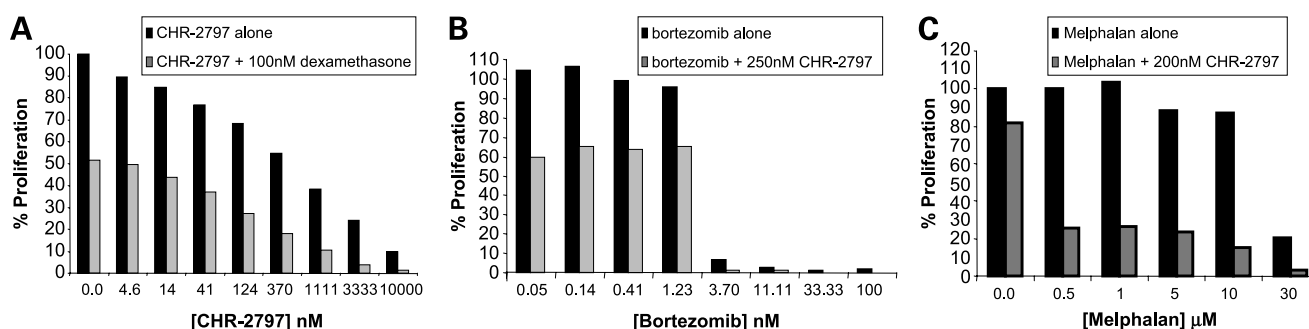
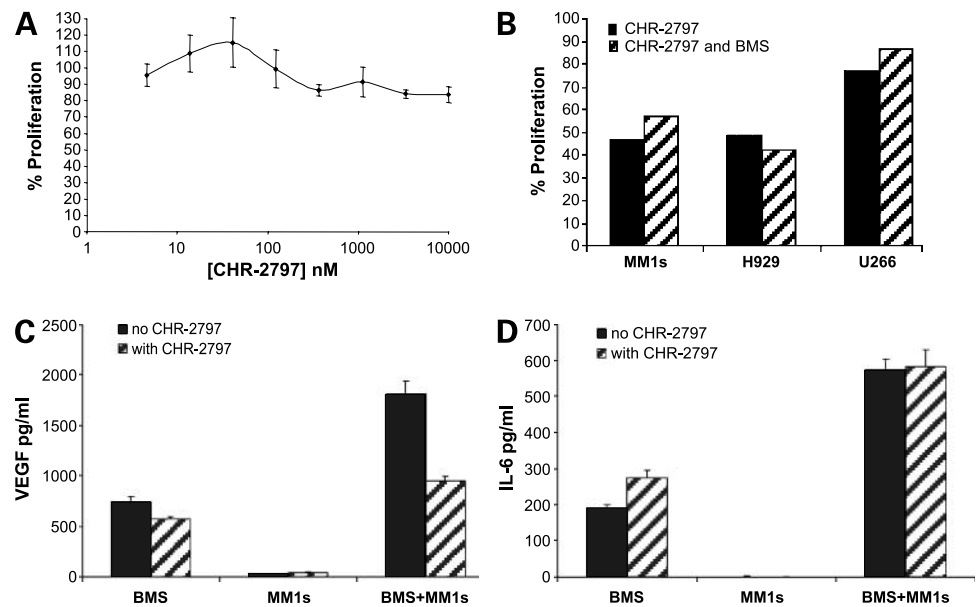


Figure 2. Combining CHR-2797 with existing myeloma therapies. **A**, combination of 100 nmol/L dexamethasone and a range of CHR-2797 doses shows synergy in the dexamethasone-sensitive cell line MM1s after 96 h. **B**, combination of CHR-2797 and bortezomib shows an additive effect when CHR-2797 is added 24 h before the bortezomib. **C**, combination of CHR-2797 and melphalan also shows synergy. Representative data are shown and all experiments were done in triplicate.

Figure 3. CHR-2797 affects BMSC/myeloma cell interactions. **A**, CHR-2797 caused minimal inhibition of proliferation of BMSCs (with a median of three different cases) over a range of doses. **B**, myeloma cell lines MM1s, H929, and U266 were cultured with CHR-2797 in the presence or absence of BMSCs for 96 h than the proliferation measured by WST-1 assay. Representative data of one of three replicates. **C** and **D**, supernatants from MM1s cells cultured in the presence/absence of BMSCs with and without CHR-2797 treatment were analyzed by ELISA for VEGF and IL-6 levels. Representative data are shown.



and R5'-CAGCCAAGCCAGAGAAGCA3', *ATF4* F5'-GCGGGCTCCTCCGAAT and R5'-GCATCCTCCTT-GCTGTTGTTG, and β -*ACTIN* F5'-CCCTGGCACCCAG-CAC and R5'-GCCGATCCACACGGAGTAC. Thermal cycling conditions were 10 min at 95°C, 40 cycles at 95°C for 15 s followed by 1 min at 60°C. Data analysis was completed using the 7500 Sequence Detection software (Applied Biosystems).

To determine relative expression levels of *XBPI/XBP1s* within a sample RT-PCR was done in a 50 μ L platinum *Taq* reaction containing 4 pmol primers (F5'-CCTTGTAGTT-GAGAACCAGG-3' and R5'-GGGGCTTGGTATA-TATGTGG-3'; Invitrogen), 1 unit Platinum *Taq* DNA polymerase, and 200 μ mol/L deoxynucleotide triphosphate. The temperature profile was at 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 60°C for 1 min, and 72°C for 30 s. Products were run on 2% agarose gels containing ethidium bromide. RT-PCR was also done to confirm these results. Primers were *XPB-1* R5'-CCAGAATGCCCAACAGGATA, F5'-SPliced CACCTCTGAGTCCGCAGCAGG[FAM]G, and F5'-UNSPliced CAGAGGCTCAGACTACGTG-CACCTC[JOE]G and thermal cycling conditions were as above.

Immunoblotting

Protein was also extracted from myeloma cells following drug treatment. Cells were centrifuged at 1,200 rpm for 5 min, washed with ice-cold PBS, pelleted, and resuspended in 1 mL ice-cold PBS. The suspension was spun at 3,500 rpm for 15 min at 4°C in a bench-top centrifuge. The supernatant was discarded, and the pellet was resuspended in lysis buffer [1% NP-40, 20 mmol/L Tris (pH 8), 100 mmol/L NaCl, 1 mmol/L EDTA] supplemented with 1 \times protease inhibitor cocktail, 50/17 mmol/L NaF, 200/2.3 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L Na₃VO₄ (Sigma-Aldrich). Nuclear and cytoplasmic fractions of protein

lysates harvested for the detection of ATF6 were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce Biotechnology). Protein concentration was measured using a BCA protein assay (Pierce Biotechnology) and 5 μ g were resolved by SDS-PAGE. This was transferred to polyvinylidene fluoride membranes, blocked with 5% milk or 5% bovine serum albumin, and incubated with primary antibody. Primary antibodies were heat shock protein 70 (Stressgen, Bioreagents), Endo G (Axxora Alexis), Noxa (Calbiochem), caspase-3, caspase-8, caspase-9, AIF, Puma, and Mcl-1 (Cell Signaling Technology), ATF6 (Stratagene), LC3 (Medical and Biological Laboratories), β -actin (Sigma-Aldrich), and CBP(A-22) (Santa Cruz Biotechnology). Secondary antibodies used were anti-mouse, anti-rabbit, or anti-rat conjugated to horseradish peroxidase (Amersham Biosciences), and Enhanced Chemiluminescence Plus (Amersham Biosciences) was used for detection. Imaging was done using Kodak XAR film and X-ray developer.

Results

CHR-2797 Inhibits Myeloma Cell Proliferation and Induces Apoptosis

CHR-2797 was shown to inhibit proliferation of all myeloma cells over a 96 h period with varying degrees of potency using a WST-1 assay (Fig. 1A). MM1s cells were the most sensitive with an IC₅₀ of 450 nmol/L followed by H929 IC₅₀ = 650 nmol/L, JLN3 IC₅₀ = 1.5 μ mol/L, and U266 IC₅₀ = 6.5 μ mol/L. Importantly MM1r, a cell line known to be resistant to the standard treatment for myeloma, dexamethasone, was sensitive to treatment with CHR-2797 with an IC₅₀ of 350 nmol/L. CHR-79888, the acid metabolite with low membrane permeability, was used as a negative control and failed to induce myeloma cell death (Fig. 1B). CD138⁺ plasma cells from patients were also sensitive to CHR-2797

treatment with an IC_{50} of 2 $\mu\text{mol/L}$ at 48 and 72 h post-treatment (Fig. 1C).

To determine whether the action of CHR-2797 was cytotoxic or cytostatic, the extent of cell death was measured by Annexin V/PI binding and trypan blue staining. The proportion of cells positive for Annexin V binding on the cell surface increased over a 96 h time course, indicating the initiation of apoptosis (increasing from 11.0% to 48.6% over the

period). This was confirmed by the increase in the percentage of cells stained with trypan blue (from 13% to 56%), although this was to a lesser extent than the inhibition of proliferation seen in the WST-1 assay and was only observed at later time points (Fig. 1D). In keeping with the above results after treatment for 24 h with CHR-2797, cell cycle analysis showed the percentage of cells in the sub- G_1 phase (indicative of apoptosis) increased in H929 from 2.5% (untreated) to 33.6% (Fig. 1E). Similar results were seen in U266 and suggest that CHR-2797 has cytotoxic activity in multiple myeloma cell lines.

CHR-2797 is Synergistic with Other Antimyeloma Therapies

To determine whether the antimyeloma effect of CHR-2797 could be increased, the compound was tested in combination with existing myeloma treatments. The dexamethasone-sensitive MM1s cells were tested with a range of CHR-2797 doses in combination with 100 nmol/L dexamethasone (Fig. 2A). The effect of the drugs in combination was greater than the effect of the drugs alone and fulfilled the formal criteria for synergism as described by Chou (17). CHR-2797 was also tested in combination with bortezomib. MM1s, U266, and H929 cells were treated with a range of bortezomib doses (100-0.05 nmol/L) in combination with 250 nmol/L CHR-2797 over 24 h (Fig. 2B). At low doses of bortezomib, the amount of inhibition of proliferation was similar to that observed with CHR-2797 alone, whereas at higher doses of bortezomib, due to the steepness of the bortezomib curve, the effects could not be differentiated from those of bortezomib alone. A full Chou-Talalay analysis with 16 points on the curve using MM1s cells showed that treating with CHR-2797 and bortezomib concomitantly, adding CHR-2797 24 h before bortezomib, or adding bortezomib 24 h before CHR-2797 yielded average CI values in the 50% fraction of 1.3075, 1.276, and 1.118, respectively, which would suggest an additive effect only when CHR-2797 was added first. Similar results were seen with the combination of CHR-2797 and the alkylating agent melphalan (Fig. 2C).

Amino peptidase Inhibition Affects BMSC/Myeloma Cell Interactions

Over the last few years, the importance of the interactions between myeloma cells and the bone marrow stroma for potentiating myeloma cell growth, survival, and

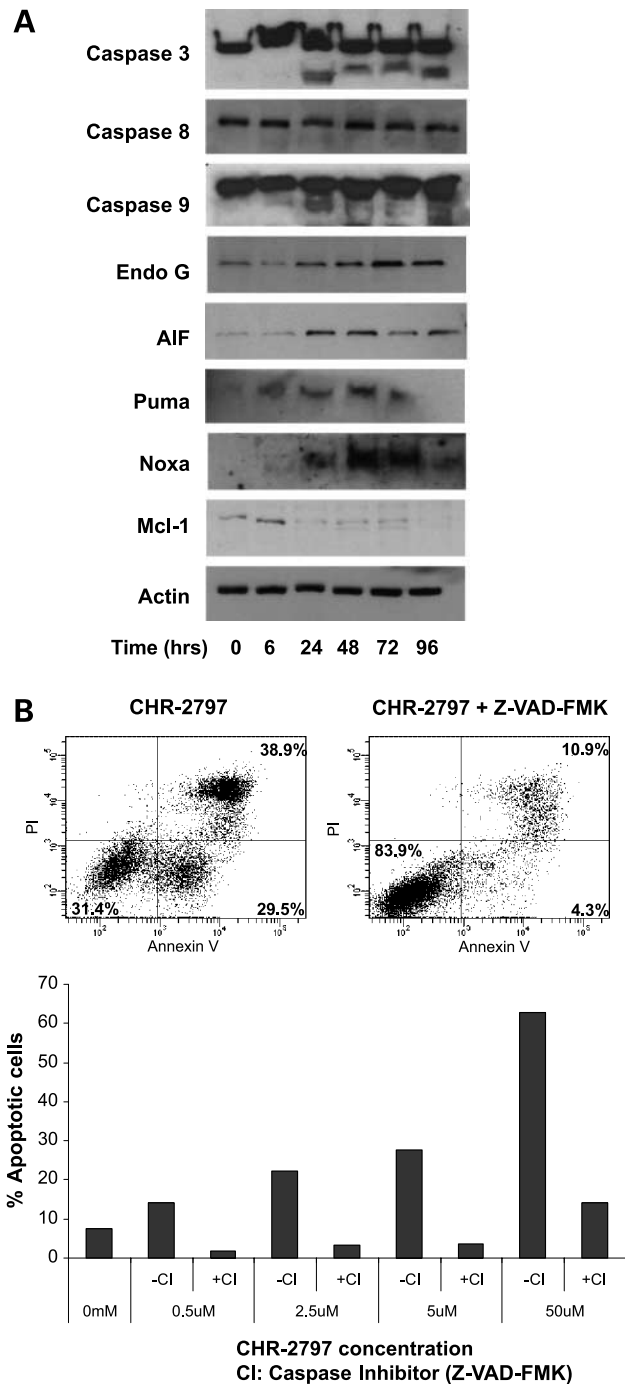
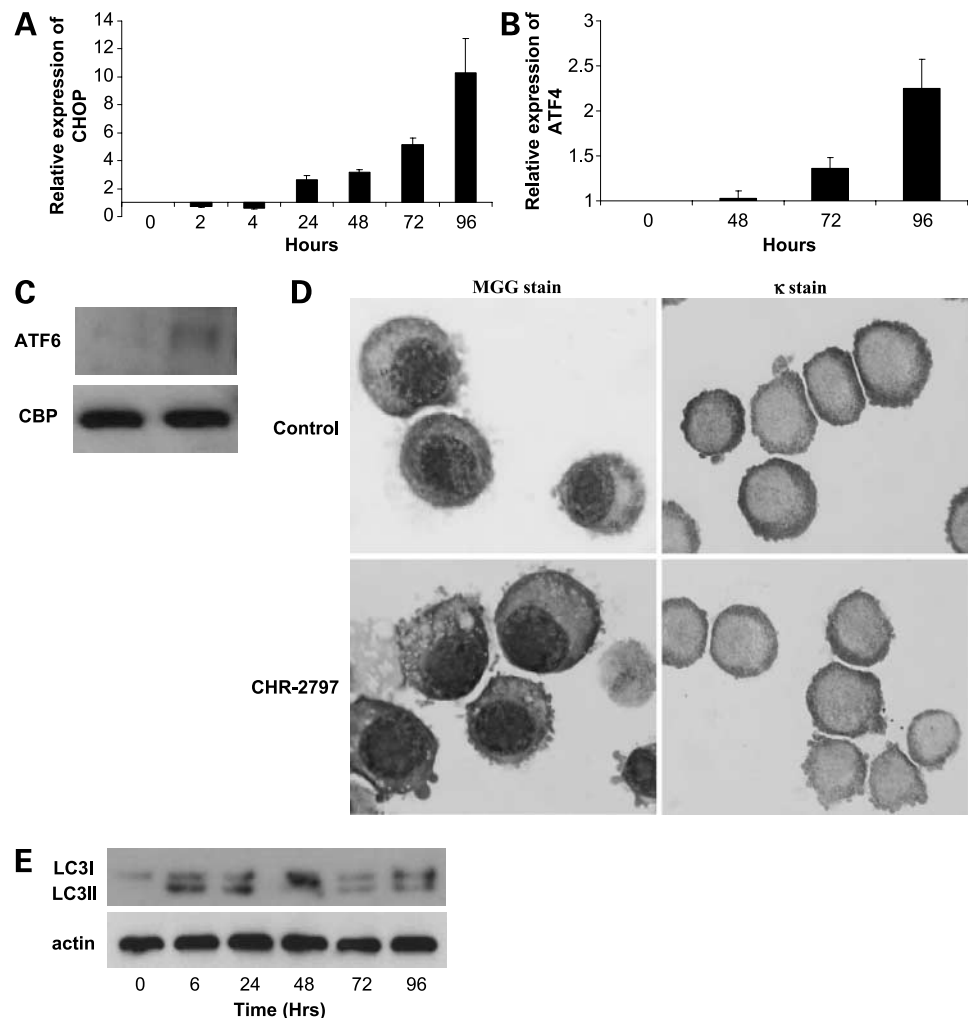


Figure 4. CHR-2797 induces myeloma cell death via caspase-dependent and caspase-independent mechanisms. **A**, Western blot analysis over a 96 h time course shows cleavage of caspase-9 and caspase-3 suggestive of activation of caspase mediated cell death by CHR-2797 (650 nmol/L). In addition to caspase-mediated cell death, an increase in AIF and Endo G (consistent with activation of non-caspase apoptosis pathways) was observed and an increase in Noxa and Puma with the cleavage of Mcl-1 is also shown. **B**, incubation with the pan-caspase inhibitor (CI) Z-VAD-FMK protected H929 cells from CHR-2797-induced apoptosis determined by Annexin V/PI binding. Cells were pretreated for 1 h, 30 min with Z-VAD-FMK and CHR-2797 was added for 24 h. *Top*, representative flow cytometry data from using CHR-2797 with and without zVADfmk pretreatment; *bottom*, percentage of cells positive for Annexin V/PI binding over a range of CHR-2797 concentrations in the presence of absence of caspase inhibitor Z-VAD-FMK. Representative data from one of three experiments on H929 are shown.

Figure 5. CHR-2797 induces the UPR. **A** and **B**, treatment of myeloma cells over a 96 h period with CHR-2797 (650 nmol/L) increased the levels of the proapoptotic factor *CHOP* and *ATF4* as determined by real-time TaqMan PCR (data normalized to β -actin expression). **C**, immunoblotting of nuclear fractions of cell lysates showed the translocation of the cleaved fragment of ATF6 α to the nucleus. CBP was used to show equal loading of the nuclear fractions. **D**, extent of cytoplasmic inclusions in response to treatment (24 h, IC_{50} doses) was determined using May-Grunwald-Giemsa staining and light microscopy ($\times 100$ oil objective magnification). **E**, Western blot analysis showed cleavage of LC3I to LC3II over the same period indicative of autophagy (actin was used as the loading control). Representative data of H929 are shown (650 nmol/L CHR-2797).



protection from drug-induced apoptosis has been highlighted. Figure 3A shows that CHR-2797 caused minimal inhibition of proliferation of BMSCs from three different patients (20% inhibition of proliferation at 10 μ mol/L). Importantly, CHR-2797 was able to overcome the protective effects of the bone marrow stroma and induced an inhibition in myeloma cell proliferation at similar IC_{50} values when myeloma cells were cultured with BMSCs compared with when myeloma cells were cultured alone (Fig. 3B).

Previous studies have shown that, when myeloma cells bind to stromal cells, IL-6 and VEGF levels are increased (18). The effect of CHR-2797 on these cytokine levels in the presence and absence of CHR-2797 was therefore determined. In keeping with other studies, the IL-6 and VEGF levels were low in supernatants from myeloma cells or bone marrow stromal cells cultured alone, but levels increased when the cells were cocultured or bound together. Treatment with CHR-2797 caused no change in IL-6 levels but caused a 48% inhibition of VEGF levels in myeloma and BMSC cocultures (Fig. 3C and D).

CHR-2797 Activates Caspase-Dependent and Caspase-Independent Apoptotic Pathways

In keeping with the cell cycle data suggesting a predominantly cytotoxic effect, in a time-course experiment following treatment with CHR-2797 for up to 96 h, cleavage of caspase-9 and caspase-3 was shown (Fig. 4A). In agreement with this finding, the proportion of cells in early or late apoptosis (determined by positive Annexin V/PI binding) was inhibited across a range of CHR-2797 concentrations by zVADfmk, a pan-caspase inhibitor (Fig. 4B). For example, following treatment with 50 μ mol/L CHR-2797 for 24 h, the percentage of cells undergoing apoptosis was 68% but decreased to 15% following the addition of zVADfmk.

In addition, we also observed an increase in the caspase-independent apoptotic proteins AIF and Endo G at 48 h, which was maintained until 96 h (Fig. 4A). A significant increase in the proapoptotic bcl-2 family members Puma and Noxa were also shown by 24 h. CHR-2797 also caused cleavage of the antiapoptotic Mcl-1 protein (Fig. 4A), an event that promotes cell death. All these findings are

consistent with involvement of both caspase-dependent and caspase-independent pathways in CHR-2797-induced apoptosis of myeloma.

Aminopeptidase Inhibition Induces the UPR and Autophagy

Our studies and those of others have shown that interfering with protein turnover using proteasome or heat shock protein inhibition is able to induce the UPR in myeloma cells, with splicing of XBP1 to XBP1s indicative of IRE1 activation, up-regulation of CHOP expression indicative of PERK activation, and activation of ATF6 (7). As aminopeptidase inhibition also interferes with protein turnover, the effects of CHR-2797 on the UPR were determined. In H929 cells, we showed using quantitative RT-PCR, increases in the expression of CHOP (Fig. 5A) and ATF4 (Fig. 5B), but no alterations in the ratio of spliced to unspliced XBP-1 transcripts (confirmed by RT-PCR; data not shown). ATF6 was also shown to be spliced to its active form, which translocates to the nucleus by 6 h (Fig. 5C). These findings suggest activation of the apoptotic branches of the UPR in response to aminopeptidase inhibition and are similar to our previous findings with the proteasome inhibitor bortezomib. However, in U266, the cell line that is more resistant to CHR-2797, the levels of CHOP remained stable, suggesting that CHR-2797 is able to induce the UPR in some cell lines but not others (data not shown). A compensatory increase in heat shock protein 70 protein levels were seen following treatment with CHR-2797, similar to the effects seen following bortezomib and 17AAG exposure, and compatible with CHR-2797 increasing cellular stress within the plasma cells (data not shown).

We have shown previously the buildup of immunoglobulin within intracellular inclusions, by light chain microscopy, following the exposure of myeloma cells to heat shock protein 90 inhibition, which disrupts protein folding. We next determined whether aminopeptidase inhibition caused similar effects and show that, following treatment of myeloma cell lines with CHR-2797 for 24 h, the formation of cellular inclusions that stain for light chain is formed (Fig. 5D). These inclusions form part of a mechanism that allows the myeloma cell to compensate for the stress induced by aminopeptidase inhibition and results in the buildup of misfolded proteins that are unable to be correctly degraded by the ubiquitin/proteasome pathway. In keeping with this, Western blot analysis shows that LC3I is cleaved to LC3II at 6 h and is maintained until 96 h, suggestive of the induction of autophagy (Fig. 5E).

Gene Expression Profiling

To gain insights into the mechanism by which CHR-2797 induces apoptosis, we performed gene expression analysis on myeloma cell lines following treatment with the IC₅₀ of dose CHR-2797 at a 24 h time point and an earlier (4 h) time point. In keeping with the early time points chosen (before the initiation of apoptosis), the majority of gene expression changes, although significant, were small with values between 1.5- and 2-fold. Treatment with CHR-2797 caused a minimal increase in *puromycin-sensitive aminopeptidase*, *leukotriene A₄ hydrolase*, and *leucine aminopeptidase 3*, known ami-

nopeptidase target genes, presumably via a positive feedback loop. In addition, CHR-2797 also altered the expression of genes encoding other aminopeptidases not previously suspected as being targets of CHR-2797, including *X-prolyl aminopeptidase (aminopeptidase P) 1* (Supplementary Table S1).³ As inhibition of aminopeptidases would be predicted to cause depletion in the quantity of free amino acids in the cell, we looked at the expression of genes involved in the synthesis of new amino acids or in the transport of amino acids into the cell. *Asparagine synthetase*, which catalyses the conversion of glutamine and aspartic acid into asparagine, was increased, consistent with reports showing an up-regulation in conditions of amino acid deprivation (19). In addition, we found that several members of *solute carrier families 1, 7, 36, and 38* that are amino acid transporters were also up-regulated in response to CHR-2797 exposure presumably as a method of overcoming the amino acid deprivation (Supplementary Table S1).³ Several tRNA synthetases, which are responsible for the recognition and attachment of amino acids to the correct tRNA during protein translation, were also found to be up-regulated in response to CHR-2797, including *cysteinylyl-* and *isoleucine-tRNA synthetase* (Supplementary Table S1).³

In a supervised analysis, 49 genes were up-regulated in all three conditions (4 h H929, 24 h H929, and 24 h U266) and no genes were down-regulated in all three conditions (Supplementary Table S2).³ Of the up-regulated genes, 13 are related to the proteasome/ubiquitin pathway and 2 are heat shock proteins. The scaffolding protein *sequestosome-1* is also up-regulated in all three settings, and this may have significance in the proteasome/ubiquitin pathway, as this scaffold sequesters polyubiquitinated proteins and shuttles them to the proteasome for degradation (20).

When comparing the expression profiles of cells treated with the proteasome inhibitor bortezomib with cells exposed to the aminopeptidase inhibitor CHR-2797 for 24 h, several similarities were seen with 219 genes up-regulated and 91 genes down-regulated by both drugs. As both drugs target protein turnover, common deregulated genes included amino acid transporters and tRNA synthetases. Many of the up-regulated genes were members of the ubiquitin proteasome pathway and the heat shock protein family, also in keeping with similar modes of action of these drugs. Of the genes down-regulated, one that may be of importance is *caveolin-1*, which has been shown previously to colocalize with the IL-6, insulin-like growth factor-I, and VEGF receptors of myeloma cells and enhance downstream signaling, increasing growth, survival, and migration of the myeloma cell (21, 22). In contrast to treatment with CHR-2797, proteasome inhibition with bortezomib did not cause any up-regulation of the putative aminopeptidase enzymes or of the key amino acid response genes such as *asparagine synthetase*.

³ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Discussion

Interfering with protein turnover using proteasome inhibition has recently been shown to be an effective approach for the treatment of myeloma. *In vitro* studies have shown that treatment with bortezomib is able to induce myeloma cell apoptosis even in the presence of the protective environment of the bone marrow (3, 4), and clinical trials have shown the induction of complete responses and improved survival compared with conventional myeloma treatments (1). As myeloma cells are effective protein factories, producing paraprotein, targeting protein turnover in a myeloma plasma cell offers an important new therapeutic strategy.

The M1 family of aminopeptidases are the intracellular metalloenzyme targets for the aminopeptidase inhibitor CHR-2797. This family of enzymes is responsible for removing an amino acid from the NH₂ terminus of proteins, in an enzyme-specific manner, which results in a shortened half-life of the target protein. In addition, like proteasome inhibition, inhibition of aminopeptidases is able to disrupt the turnover of cell cycle intermediates and cause a reduction in the free amino acid content in the cell. All of these mechanisms are postulated to result in myeloma cell death, particularly as malignant cells are often more dependent on the availability of amino acids such as arginine. There is a precedent for taking such an approach in hematologic cancers, as bestatin, a broad-spectrum inhibitor of the aminopeptidases, has been shown to be effective in Japan in solid tumors and leukemia and is well tolerated in clinical trials (13, 14).

Our studies show that myeloma plasma cells are sensitive to aminopeptidase inhibition. Treatment with CHR-2797 is able to inhibit proliferation and induce apoptosis of myeloma cell lines and primary patient cells, with minimal effects on bone marrow stroma. Importantly, exposure to the drug is able to overcome the protective effects of the bone marrow microenvironment and inhibit the proliferation of myeloma cells even when bound to BMSCs. CHR-2797 is also able to decrease the production of VEGF caused by the binding of myeloma plasma cells to bone marrow stroma, suggesting that the effect on myeloma cell proliferation is mediated, at least in part, by the inhibition of paracrine growth and survival cytokines. As well as its effects on mediating the paracrine growth of myeloma cells, VEGF has also been shown to have several other important roles in myeloma biology. These include protecting cells from drug-induced apoptosis, mediating myeloma cell migration, and mediating bone marrow angiogenesis (18, 21, 22).

Bortezomib and CHR-2797 both interfere with protein turnover but target the pathways at different points, the proteasome and peptide breakdown, respectively (data not shown). In keeping with both drugs targeting this pathway, our experiments show there are several similarities and differences in their biological effects. The gene expression profiling shows that, as expected, treatment with the proteasome inhibitor results in the up-regulation of many genes involved in the ubiquitin proteasome pathway. Interestingly, similar effects are also seen following treatment

with CHR-2797. Both drugs also affect the levels of amino acid transporters and tRNA synthetases; however, alterations in the aminopeptidase enzymes and the amino acid response genes are only seen following treatment with CHR-2797.

Both drugs are able to inhibit myeloma cell growth and induce apoptosis of myeloma cells and importantly are able to induce apoptosis in myeloma cells in the presence of BMSCs. Bortezomib and CHR-2797 have been shown to induce apoptosis in both caspase-dependent and non-caspase-dependent pathways. Although both drugs target a similar pathway, protein turnover, the drugs exert their effects at different places in this process and this results in an additive effect when bortezomib and CHR-2797 are used in combination. Several recent studies have shown that Mcl-1 is an essential survival protein in myeloma and that cleavage of Mcl-1 and an increase in the BH3-only protein Noxa are major hallmarks of bortezomib sensitivity (23). Our study would suggest that both of these proteins are also important in mediating the apoptotic effects of CHR-2797, suggesting that Mcl-1 cleavage and Noxa up-regulation are important events seen when protein turnover is disrupted.

Importantly, the effects on protein transport and degradation caused by CHR-2797 can be shown at the cellular level with the observation of cellular inclusions containing immunoglobulin light chain, the activation of the UPR with increases in CHOP indicative of PERK activation and cleavage of ATF6, and the activation of the autophagy pathway with the cleavage of LC31 to LC3II. Our experiments would suggest that activation of these key pathways represents an adaptive phase where the myeloma cell tries to compensate for the effects of cellular stress; however, these compensatory mechanisms fail and over time apoptosis ensues.

In summary, we show that the aminopeptidase inhibitor CHR-2797 is able to inhibit myeloma cell growth and survival in several ways. These include direct effects on the myeloma cell as well as effects on the bone marrow microenvironment, suggesting that this approach may be useful therapeutically. Given the exciting results of this *in vitro* study, a phase I/II clinical trial is under way at our institution and in several other institutions in Europe, looking at the safety and clinical effectiveness of CHR-2797 in myeloma and in other hematologic malignancies (24). *In vitro* data examining the effects of CHR-2797 on solid tumors are also promising (15) and clinical trials in this area are ongoing.

Disclosure of Potential Conflicts of Interest

D. Krige, A.H. Drummond, and L. Hoofman: employees with stock options in Chroma Therapeutics Ltd.; F.E. Davies and G.J. Morgan: consultants for Chroma Therapeutics Ltd. No other potential conflicts of interest were disclosed.

Acknowledgments

We thank Alex Javed for technical expertise with the immunoglobulin light chain staining.

References

1. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med* 2003;348:2609–17.
2. Hideshima T, Chauhan D, Richardson P, et al. NF- κ B as a therapeutic target in multiple myeloma. *J Biol Chem* 2002;277:16639–47.
3. Mitsiades N, Mitsiades CS, Poulaki V, et al. Molecular sequelae of proteasome inhibition in human multiple myeloma cells. *Proc Natl Acad Sci U S A* 2002;99:14374–9.
4. Hideshima T, Mitsiades C, Akiyama M, et al. Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. *Blood* 2003;101:1530–4.
5. Landowski TH, Megli CJ, Nullmeyer KD, Lynch RM, Dorr RT. Mitochondrial-mediated dysregulation of Ca^{2+} is a critical determinant of Velcade (PS-341/bortezomib) cytotoxicity in myeloma cell lines. *Cancer Res* 2005;65:3828–36.
6. Obeng EA, Carlson LM, Gutman DM, Harrington WJ, Jr., Lee KP, Boise LH. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. *Blood* 2006;107:4907–16.
7. Davenport EL, Moore HE, Dunlop AS, et al. Heat shock protein inhibition is associated with activation of the unfolded protein response (UPR) pathway in myeloma plasma cells. *Blood* 2007;110:2641–9.
8. Moerschell RP, Hoskawa Y, Tsunasawa S, Sherman F. The specificities of yeast methionine aminopeptidase and acetylation of amino-terminal methionine *in vivo*. *J Biol Chem* 1990;265:19638–43.
9. Botbol V, Scornik OA. Measurement of instant rates of protein degradation in the livers of intact mice by the accumulation of bestatin-induced peptides. *J Biol Chem* 1991;266:2151–7.
10. Constan DB, Tobler AR, Rensing-Ehl A, Kemler I, Hersh LB, Fontana A. Puromycin-sensitive aminopeptidase. Sequence analysis, expression, and functional characterization. *J Biol Chem* 1995;270:26931–9.
11. Scott L, Lamb J, Smith S, Wheatley DN. Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. *Br J Cancer* 2000;83:800–10.
12. Wheatley DN, Scott L, Lamb J, Smith S. Single amino acid (arginine) restriction: growth and death of cultured HeLa and human diploid fibroblasts. *Cell Physiol Biochem* 2000;10:37–55.
13. Ichinose Y, Genka K, Koike T, et al. Randomized double-blind placebo-controlled trial of bestatin in patients with resected stage I squamous-cell carcinoma. *J Natl Cancer Inst* 2003;95:605–10.
14. Ota K, Uzuka Y. Clinical trials of bestatin for leukemia and solid tumors. *Biotherapy* 1992;4:205–14.
15. Krige D, Needham LA, Bawden LJ, et al. CHR-2797 an antiproliferative aminopeptidase inhibitor that leads to amino acid deprivation in human leukaemia cells. *Cancer Res* 2008;68:6669–79.
16. Walker BA, Leone PE, Jenner MW, et al. Integration of global SNP-based mapping and expression arrays reveals key regions, mechanisms, and genes important in the pathogenesis of multiple myeloma. *Blood* 2006;108:1733–43.
17. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006;58:621–81.
18. Gupta D, Treon SP, Shima Y, et al. Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. *Leukemia* 2001;15:1950–61.
19. Kilberg MS, Barbosa-Tessmann IP. Genomic sequences necessary for transcriptional activation by amino acid deprivation of mammalian cells. *J Nutr* 2002;132:1801–4.
20. Seibenhener ML, Geetha T, Wooten MW. Sequestosome 1/p62—more than just a scaffold. *FEBS Lett* 2007;581:175–9.
21. Podar K, Tai YT, Cole CE, et al. Essential role of caveolae in interleukin-6- and insulin-like growth factor I-triggered Akt-1-mediated survival of multiple myeloma cells. *J Biol Chem* 2003;278:5794–801.
22. Podar K, Shringarpure R, Tai YT, et al. Calveolin-1 is required for the vascular endothelial growth factor-triggered multiple myeloma cell migration and is targeted by bortezomib. *Cancer Res* 2004;64:7500–6.
23. Gomez-Bougie P, Wuilleme-Toumi S, Menoret E, et al. Noxa upregulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res* 2007;67:5418–24.
24. Davies F, Ossenkoppele G, Zachee P, et al. A phase I study of CHR-2797, an orally active aminopeptidase inhibitor in elderly and/or treatment refractory patients with acute myeloid leukemia or multiple myeloma. *Blood* 2007;110:abstract 443.

Molecular Cancer Therapeutics

Aminopeptidase inhibition as a targeted treatment strategy in myeloma

Hannah E. Moore, Emma L. Davenport, Emma M. Smith, et al.

Mol Cancer Ther 2009;8:762-770.

Updated version	Access the most recent version of this article at: http://mct.aacrjournals.org/content/8/4/762
Supplementary Material	Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2021/03/11/8.4.762.DC1

Cited articles	This article cites 24 articles, 16 of which you can access for free at: http://mct.aacrjournals.org/content/8/4/762.full#ref-list-1
Citing articles	This article has been cited by 3 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/8/4/762.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/8/4/762 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.